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(REV. 11-94)

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

ATTORNEY'S DOCKET NUMBER
8484-018-999

08/765244

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/DE95/00775	11 JUNE 1995	16 JUNE 1994

TITLE OF INVENTION
CHIMERICAL PEPTIDE-NUCLEIC ACID FRAGMENT, PROCESS FOR PRODUCING THE SAME AND ITS USE FOR APPROPRIATELY INTRODUCING NUCLEIC ACIDS INTO CELL ORGANELLES AND CELLS

APPLICANT(S) FOR DO/EO/US
PETER SEIBEL and ANDREA SEIBEL

Applicant herewith submits to the United States Designated/ Elected Office (DO/EO/US) the following items under 35 U.S.C. 371:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the international Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)

6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureaus.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 37(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☒ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information:

* A Verified Statement (Declaration) Claiming Small Entity Status [37 CFR 1.9(f) and 1.27(b)] - Independent Inventor

INTERNATIONAL APPLICATION NO. PCT/DE95/00775	INTERNATIONAL FILING DATE 11 JUNE 1995

17. ☒ The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees as follows:

CLAIMS				
(1)FOR	(2)NUMBER FILED	(3)NUMBER EXTRA	(4)RATE	(5)CALCULATIONS
TOTAL CLAIMS	71 -20=	51	X $ 22.00	$ 1,122.00
INDEPENDENT CLAIMS	2 -3=	0	X $ 80.00	0.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $ 260.00	260.00

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): **CHECK ONE BOX ONLY**	
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) . . . . . $ 700	
☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) . . . . . $ 770	
☒ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $1040	1040.00
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2) to (4) . . . . . $ 96	
☐ Filing with EPO or JPO search report . . . . . $ 910	
Surcharge of $130.00 for furnishing the National fee or oath or declaration later than ☐ 20 ☒ 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).	130.00
TOTAL OF ABOVE CALCULATIONS =	2,552.00
Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (Note 37 CFR 1.9, 1.27, 1.28). -	1,276.00
SUBTOTAL =	1,276.00
Processing fee of $130.00 for furnishing the English Translation later than ☐ 20 ☒ 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)). +	130.00
TOTAL FEES ENCLOSED $	1,406.00

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge Deposit Account No. 16-1150 in the amount of $ 1406.00 to cover the above fees. A copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-1150. A copy of this sheet is enclosed.

18. ☒ Other instructions

Please calculate fees for the claims after entering the first Preliminary Amendment.

19. ☒ All correspondence for this application should be mailed to

PENNIE & EDMONDS
1155 AVENUE OF THE AMERICAS
NEW YORK, NEW YORK 10036-2711

20. ☒ All telephone inquiries should be made to (212) 790-2803

Albert P. Halluin	Albert P. Halluin	25,227	12/16/96
NAME	SIGNATURE	REGISTRATION NUMBER	DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: ☒ Application of: Seibel *et al.*
☐ Patent of:

☒ Serial No.:
☐ Patent No.:

Group Art Unit: To Be Assigned

☒ Filed: December 16, 1996
☐ Issued:

Examiner: To Be Assigned

For: Chimerical Peptide-Nucleic Acid Fragment, Process For Producing The Same And Its Uses For Appropriately Introducing Nucleic Acids Into Cell Organelles and Cells

Attorney Docket No.: 8484-018-999

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
[37 CFR 1.9(f) and 1.27(b)] - Independent Inventor

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled Chimerical Peptide-Nucleic Acid Fragment, Process For Producing The Same And Its Uses For Appropriately Introducing Nucleic Acids Into Cell Organelles and Cells described in

☐ the specification filed herewith
☒ application serial no. _____ filed December 16, 1996
☐ patent no. issued

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

☒ no such person, concern, or organization
☐ persons, concerns or organizations listed below*

*NOTE: Separate verified statements are required from each named person, concern, or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME__________
ADDRESS__________

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

FULL NAME__________
ADDRESS__________

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

FULL NAME__________
ADDRESS__________

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

FULL NAME__________
ADDRESS__________

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28 (b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, and patent issuing thereon, or any patent to which this verified statement is directed.

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NAME OF INVENTOR Peter Seibel	NAME OF INVENTOR Andrea Seibel
SIGNATURE OF INVENTOR Peter Seibel	SIGNATURE OF INVENTOR Andrea Seibel
DATE 21.3.97	DATE 21.3.97

PEMP-68065.1

08/765244
52 Rec'd PCT/PTO DEC 16 1996

Express Mail No. TB 665 381 717 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Peter Seibel et al.

Serial No.: UNASSIGNED

Group Art Unit: UNASSIGNED

Filed: HEREWITH

Examiner: UNASSIGNED

For: Chimerical Peptide-Nucleic Acid Fragment, Process for Producing the Same and Its Use For Appropriately Introducing Nucleic Acids Into Cell Organelles and Cells

Attorney Docket No.: 8484-018-999

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In accordance with Rule 115 of the Rules of Practice, 37 C.F.R. § 1.115, please consider and enter the following amendments and remarks.

IN THE CLAIMS:

1. A chimerical peptide-nucleic acid fragment comprising:

(a) a cell-specific, compartment-specific or membrane-specific signal peptide, with the exception of a KDEL signal sequence,

(b) a linkage agent,

(c) a nucleic acid (oligonucleotide),

the signal peptide being linked via the linkage agent which via amino acids at the carboxy-terminal end of the signal peptide is linked therewith so as to ensure the appropriate nucleic acid introduction into cell organelles and cells.

2. (once amended). The chimerical peptide-nucleic acid fragment according to claim 1, [characterized in that]wherein the nucleic acid consists of at least two bases.

3. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s 1 to] 2, [characterized in that]wherein the nucleic acid has a secondary structure.

4. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s 1 to 3]2, [characterized in that]wherein the nucleic acid has a palindromic sequence.

5. (once amended). The chimerical peptide-nucleic acid fragment according to claim 4, [characterized in that]wherein the nucleic acid may form a "hairpin loop".

6. (once amended). The chimerical peptide-nucleic acid fragment according to claim 5, [characterized in that]wherein the nucleic acid may hybridize with itself and may form an overhanging 3' end or 5' end ('sticky end').

7. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 1[to 6], [characterized in that]wherein the nucleic acid is a ribonucleic acid, preferably a deoxyribonucleic acid.

8. (once amended). The chimerical peptide-nucleic acid fragment according to claim 7, [characterized in that]wherein the nucleic acid has chemically modified 'phosphorus thioate' linkages.

9. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 1[to 8], [characterized in that]wherein the nucleic acid carries a reactive linkage group.

10. (once amended). The chimerical peptide-nucleic acid fragment according to claim 9, [characterized in that]wherein the reactive linkage group contains an amino function when the linkage agent contains an amino-reactive grouping.

11. (once amended). The chimerical peptide-nucleic acid fragment according to claim 9, [characterized in that]wherein the reactive linkage group contains a thiol function when the linkage agent contains a thiol-reactive grouping.

12. (once amended). The chimerical peptide-nucleic acid fragment according to claim 10 or 11, [characterized in that]wherein the linkage grouping present is bound to the nucleic acid via at least one C2 spacer, but preferably one C6 spacer.

13. (once amended). The chimerical peptide-nucleic acid fragment according to claim 12, [characterized in that]wherein the linkage grouping is localized at the 3' hydroxy/phosphate terminus or at the 5' hydroxy/phosphate terminus of the nucleic acid, but preferably at the base.

14. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s 10 to 13]12, [characterized in that]wherein defined nucleic acids, antisense oligonucleotides, messenger RNAs or transcribable and/or replicatable genes are linked with the 5' end and/or 3' end.

15. (once amended). The chimerical peptide-nucleic acid fragment according to claim 14, [characterized in that]wherein the nucleic acid to be linked contains chemically modified 'phosphorus thioate' linkages.

16. (once amended). The chimerical peptide-nucleic acid fragment according to claim 14[to 15], [characterized in that]wherein the gene be linked contains a promotor, preferably a mitochondrial promoter.

17. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 1[to 16], [characterized in that]wherein the signal peptide has a reactive amino acid at the carboxy-terminal end, preferably a lysine or cysteine, when the linkage agent contains an amino-reactive or thiol-reactive grouping.

18. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 1[to 17], [characterized in that]wherein the signal peptide carries a cell-specific, compartment-specific or membrane-specific recognition signal.

19. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 1[to 18], [characterized in that]wherein the signal peptide has a cell-specific, compartment-specific or membrane-specific peptidase cleavage site.

20. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 1[to 19], [characterized in that]wherein the peptide consists of the compartment-specific cleavable signal peptide of the human mitochondrial ornithine transcarbamylase, extended by an artificial cysteine at the C terminus.

21. (once amended). The chimerical peptide-nucleic acid fragment according to any one of claim[s] 1[to 20], [characterized in that]wherein the linkage agent is a bifunctional, preferably heterobifunctional cross-linker.

22. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 1[to 21], [characterized in that]wherein the linkage agent contains thiol-reactive and/or amino-reactive groupings when the signal peptide and the nucleic acid carry thiol and/or amino groups as linkage sites.

23. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 1[to 22], [characterized in that]wherein the linkage agent is m-maleimido-benzoyl-N-hydroxy-succinimide ester or a derivative thereof.

24. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 1[to 23], [characterized in that]wherein the molecule can overcome membranes with and without membrane potential by utilizing natural transport mechanisms.

25. (once amended). [The]A chimerical peptide-nucleic acid fragment in the form of a linear-cyclic plasmid, [characterized in that]wherein the plasmid comprises at least one

replication origin and that both ends of the nucleic acid portion are cyclized, at least one cyclic end having a modified nucleotide which via a linkage agent can be liked with a cell-specific, compartment-specific or membrane-specific signal peptide.

26. (once amended). The chimerical peptide-nucleic acid fragment according to claim 25, [characterized in that]wherein the nucleic acid portion further comprises at least one promoter, preferably a mitochondrial promoter, especially preferably the mitochondrial promoter of the light strand.

27. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[and 26], [characterized in that]wherein the nucleic acid portion further comprises transcription-regulatory sequences, preferably mitochondrial transcription-regulatory sequences.

28. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[-27], [characterized in that]wherein the transcription-regulatory sequences have at least one binding site of a transcription initiation factor.

29. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] Claim[s] 25[to 28], [characterized in that]wherein the transcription-regulatory sequences have at least one binding site for the RNA synthesis apparatus, preferably the binding site for the mitochondrial transcription factor 1 and the mitochondrial RNA polymerase.

30. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 29], [characterized in that]wherein the transcription-regulatory sequences are arranged in the 3' direction of the promoter.

31. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 30], [characterized in that]wherein the transcription is regulated by elements of the mitochondrial H-strand and L-strand transcription control.

32. (once amended). The chimerical peptide-nucleic acid fragment according to claim 31, [characterized in that what is called]wherein the 'conserved-sequence-blocks' of L-strand transcription act as transcription control elements.

33. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 32], [characterized in that]wherein the plasmid further comprises at least one transcription termination site.

34. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 33], [characterized in that]wherein the transcription termination site has a binding sequence of a mitochondrial transcription termination factor.

35. (once amended). The chimerical peptide-nucleic acid fragment according to claim 34, [characterized in that]wherein the transcription termination site has the binding sequence of a preferably bidirectionally acting transcription termination factor.

36. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 35], [characterized in that]wherein the replication origin is a mitochondrial replication origin, preferably the replication origin of the heavy mtDNA strand having at least one 'conserved sequence block'.

37. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 36], [characterized in that]wherein the plasmid further comprises at least one regulatory sequence for the replication.

38. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 37], [characterized in that]wherein the regulatory sequence for the replication is a mitochondrial sequence motif.

39. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 38], [characterized in that]wherein the plasmid further comprises a selection gene, preferably an antibiotic-resistance gene, preferably the oligomycin - or chloramphenicol - resistance gene.

40. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 39], [characterized in that]wherein the plasmid further contains a multiple cloning site which permits the expression of 'foreign genes'.

41. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 40], [characterized in that]wherein the multiple cloning site comprises recognition sequences for restriction endonucleases which do preferably not occur in another site of the plasmid.

42. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 41], [characterized in that]wherein the multiple cloning site is arranged in the 3' direction of the promoter and in the 5' direction of the transcription termination site.

43. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 42], [characterized in that]wherein the multiple cloning site is arranged in the 5' direction of the selection gene.

44. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 43], [characterized in that]wherein the nucleic acid fragment has (phosphorylated) ends capable of litigation.

45. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 44], [characterized in that]wherein the nucleic acid fragment has 'blunt ends' or overhanging 3' ends, preferably overhanging 5' ends.

46. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 45], [characterized in that]wherein the nucleic acid fragment has 4 nucleotides comprising 5' overhangs which do not have a self-homology (palindromic sequence) and are not complementary to one another either.

47. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 46], [characterized in that]wherein the ends of the nucleic acid fragment are cyclized via synthetic oligonucleotides.

48. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 47], [characterized in that]wherein the overhanging 5' ends of the two oligonucleotides are complementary to one differing end of the nucleic acid each.

49. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 48], [characterized in that]wherein two differing 'hairpin loops' are used for the cyclization, one being specific (complementary) to the 'left' plasmid end and the other being specific to the 'right' plasmid end of the nucleic acid.

50. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 49], [characterized in that]wherein the modified nucleotide is localized preferably within the 'loop'.

51. (once amended). The chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 25[to 50], [characterized in that]wherein the plasmid DNA is amplified enzymatically by suitable oligonucleotides which have at least one recognition sequence for a restriction endonuclease which occurs preferably in non-repeated fashion in the plasmid sequence.

52. (once amended). The chimerical peptide-nucleic acid fragment according to claim 51, [characterized in that]wherein the restriction endonuclease to be used generated overhanging ends, preferably 5' overhanging ends, the cleavage site being localized preferably outside the recognition sequence.

53. (once amended). The chimerical peptide-nucleic acid fragment according to claim 51[or 52], [characterized in that]wherein the restriction endonuclease is B*sa*I.

54. (once amended). A process for the production of a chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 1[to 53]or 25, [characterized by]comprising the following stages:

(a) reaction of a nucleic acid (oligonucleotide) containing a functional linkage group having a linkage agent,

(b) reaction of the construct of (a) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL signal sequence, and

(c) optionally extension of the chimerical peptide-nucleic acid fragment of (b) by further DNA or RNA fragments.

55. (once amended). The process according to claim 54, [characterized in that]wherein the DNA in step (c) is a PCR-amplified DNA fragment containing the human mitochondrial promoter of the light strand (P_L) as well as the gene for the mitochondrial transfer RNA leucine ($tRNALeu^{UUR)}$).

56. (once amended). The process for the production of a chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 1[to 53]or 25, [characterized by]comprising the following steps:

(a) optional extension of the nucleic acid containing a functional linkage group by further DNA or RNA fragments,

(b) reaction of the nucleic acid with functional linkage group or the extended nucleic acid of (a) with a linkage agent,

(c) reaction of the construct of (b) with amino acids at the carboxy-terminal end of a peptide containing a signal sequence, with the exception of a KDEL signal sequence.

57. (once amended). The process according to claim 56, [characterized in that]wherein the DNA in step (a) is a PCR-amplified DNA fragment containing the human mitochondrial promoter of the light strand (P_L) as well as the gene for the mitochondrial transfer RNA leucine ($tRNALeu^{UUR)}$).

58. (once amended). [Use of]A method to use the chimerical peptide-nucleic acid fragment according to[any one of] claim[s] 1[to 53]or 25 for the appropriate nucleic acid introduction into cell organelles and cells, [characterized by]comprising reacting the fragment with cells or pretreated cell compartments.

59. (once amended). [Use]The method according to claim 58, [characterized in that]wherein the pretreated cell compartments are energized mitochondria.

60. (once amended). [Use of]A method of using the chimerical peptide-nucleic acid fragment according to[any one of] claims 1 [to 59]or 25 for the introduction into eukaryotic cells.

61. (once amended). [Use of a chimerical peptide-nucleic acid fragment]The method according to claim 60, [characterized by]comprising employing] the 'particle gun' system, electroporation, microinjection or lipotransfection for the introduction into eukaryotic cells.

REMARKS

The above amendments are made to comply with the formal requirements set forth in 37 C.F.R. §1.75. They do not introduce new matter, and they are fully supported by the specification of the subject Application.

Applicants respectfully request that the above-made amendments be made of record in the file history of the instant application.

Respectfully submitted,

Date 12/16/96

ALBERT P. HALLUIN 25,227 (Reg. No.)

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Enclosure

08765514 . 120397

08/765244
27 Rec'd FCT/PTC 16 DEC 1996

Chimerical peptide-nucleic acid fragment, process for producing the same and its use for appropriately introducing nucleic acids into cell organelles and cells

This invention relates to a chimerical peptide-nucleic acid fragment, the process for producing the same and its use for appropriately introducing nucleic acids into cell organelles and cells.

It is known that cellular membrane systems are largely impermeable to nucleic acids. However, cell membranes can be overcome very efficiently by physical processes (transformation) and biological processes (infection). Transformation, i.e. the direct absorption of the naked nucleic acid by the cell, is preceded by cell treatment. There are various methods available for the production of these 'competent cells'. Most processes are based on the observations made by Mandel and Higa (M. Mandel *et al.*, (1970), "Calcium-dependent bacteriophage DNA infection", J. Mol. Biol. 53: 159-162), who could show for the first time that the yields resulting from the absorption of lambda-DNA by bacteria can be increased fundamentally in the presence of calcium chloride. This method is also used successfully for the first time by Cohen *et al.* (S.N. Cohen *et al.* (1972), "Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA", Proc. Natl. Acad. Sci. U.S.A. 69: 2110-2114) for plasmid DNA and was improved by many modifications (M. Dagert *et al.* (1979), "Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells", Gene 6: 23-28). Another transformation method is based on the observation that high-frequency alternating fields may break up cell membranes (electroporation). This technique can be used to introduce naked DNA into not only prokaryotic cells but also eukaryotic cell systems (K. Shigekawa *et al.* (1988), "Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells", Biotechniques 6: 742-751). Two very gentle methods of introducing DNA into eukaryotic cells were developed by Capecchi (M.R. Capecchi (1980),

"High efficiency transformation by direct microinjection of DNA into cultured mammalian cells" Cell 22: 479-488) and Klein *et al.* (T.M. Klein *et al.* (1987), "High velocity microprojectiles for delivering nucleic acids into living cells", Nature 327: 70-73): They are based on the direct injection of the DNA into the individual cell (microinjection), on the one hand, and on the bombardment of a cell population with microprojectiles consisting of tungsten, to the surface of which the corresponding nucleic acid was bound ('shotgun'). The biological infection methods proved their value parallel to the physical transformation of cells. They include particularly the highly efficient viral introduction of nucleic acids into cells (K.L. Berkner (1988), "Development of adenovirus vectors for the expression of heterologous genes", Biotechniques 6: 616-629; L.K. Miller (1989), "Insect baculoviruses: powerful gene expression vectors", Bioessays 11:91-95; B. Moss *et al.* (1990), "Product review. New mammalian expression vectors", Nature 348: 91-92) and the liposome mediated lipofection (R.J. Mannino *et al.* (1988), "Liposome mediated gene transfer", Biotechniques 6: 682-690; P.L. Felgner *et al.* (1987), "Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure", Proc. Natl. Acad. Sci. U.S.A. 84: 7413-7417). All methods described so far deal with the overcoming of the prokaryotic or eukaryotic plasma membrane by naked or packaged nucleic acids. While the site of action is reached already when the nucleic acid are introduced into the prokaryotic cell, further biochemical processes take place in a compartmentalized eukaryotic cell, which support the penetration of the nucleic acid into the nucleus under certain conditions (e.g. viral route of infection in the case of HIV). Analogous infective processes in which exogenous nucleic acids are actively introduced into other cell organelles (e.g. into mitochondria) have not been described so far.

In addition to the introduction of the nucleic acid into the cell and cell organelle, respectively, the

transcription and above all the replication of the introduced nucleic acid play a decisive part. In this connection, it is known that the DNA molecules may have a special property which permits duplication in a cell under certain conditions. A special structural element, the origin of the DNA replication (ori, origin), adds thereto. Its presence provides the ability of DNA replication (K.J. Marians (1992), "Prokaryotic DNA replication", Annu. Rev. Biochem. 61: 673-719; M.L. DePamphilis (1993), "Eukaryotic DNA replication: anatomy of an origin", Annu. Rev. Biochem. 62: 29-63; H. Echols and M.F. Goodman (1991), "Fidelity mechanisms in DNA replication", Annu. Rev. Biochem. 60: 477-511). The strictly controlled process of DNA replication starts in E. coli e.g. when a protein is bound (K. Geider and H. Hoffmann Berling (1981), "Proteins controlling the helical structure of DNA", Annu. Rev. Biochem. 50: 233-260) to the highly specific initiation site thus defining the starting point of a specific RNA polymerase (primase). It synthesizes a short RNA strand (~ 10 nucleotides, 'primer') which is complementary to one of the DNA template strands. The 3' hydroxyl group of the terminal ribonucleotide of this RNA chain serves as 'primer' for the synthesis of new DNA by a DNA polymerase. DNA-untwisting proteins unwind the DNA double helix (J.C. Wang (1985), "DNA topoisomerases", Annu. Rev. Biochem. 54: 665-697). The separated individual strands are stabilized by DNA-binding proteins as regards their conformation (J.W. Chase and K.R. Williams (1986), "Single-stranded DNA binding proteins required for DNA replication", Annu. Rev. Biochem 55: 103-136) to enable proper functioning of the DNA polymerases (T.S. Wang (1991), "Eukaryotic DNA polymerases", Annu. Rev. Biochem. 60: 513-552). A multienzyme complex, the holoenzyme of DNA-polymerase-III, synthesizes the majority of the new DNA. The RNA portion of the chimerical RNA-DNA molecule is then split off the DNA polymerase III. The removal of the RNA from the newly formed DNA chains creates gaps between the DNA fragments. These gaps are filled by the DNA-polymerase I which can newly synthesize DNA from a single-stranded template. While

one of the two newly synthesized DNA strands is synthesized continuously (5'-3' direction, leader strand), Ogawa and Okazaki observed that a majority of the newly synthesized opposite strand (3'-5' direction, delayed strand) was synthesized from short DNA fragments (T. Ogawa and T. Okazaki (1980), "Discontinuous DNA replication", Annu. Rev. Biochem. 49: 421-457). Here, what is called primases initiate the onset of the DNA synthesis of the opposite strand by the synthesis of several RNA primers. When the replication proceeds, these fragments are freed from their RNA primers, the gaps are closed and covalently linked with one another to give extended daughter strands by the DNA ligase. Two chromosomes form after the termination of the replication cycle.

As opposed thereto, the DNA replication is controlled by many plasmids via a replication origin which dispenses with the synthesis of the delayed strand (3'-5' direction) and can initiate the synthesis of two continuous DNA strands bidirectionally (each in the 5'-3' direction along the two templates). The precondition for a complete DNA replication is here the cyclic form of the nucleic acid. It ensures that at the end of the new synthesis of the complementary DNA strands the DNA polymerases return to the starting point again where now ligases guarantee the covalent linkage of the ends of the two newly synthesized daughter strands.

Smallpox viruses represent an interesting form of linear-cyclic nucleic acids: because of what is called 'hairpin loops' at the ends of their linear genomes they have a cyclic molecule structure while maintaining a predominantly linear conformation (D.N. Black *et al.* (1986), "Genomic relationship between capripoxviruses", Virus Res. 5: 277-292; J.J. Esposito and J.C. Knight (1985) "Orthopoxvirus DNA: a comparison of restriction profiles and maps", Virology 143: 230-251). Covalently closed 'hairpin' nucleic acids were not only found in smallpox viruses but also described for the ribosomal RNA from Tetrahymena (E.H.

Blackburn and J.G. Gall (1978), "A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena", J. Mol. Biol. 120: 33-53) and the genomes of the parvoviruses (S.E. Straus *et al.* (1976), "Concatemers of alternating plus and minus strands are intermediates in adenovirus-associated virus DNA synthesis", Proc. Natl. Acad. Sci. U.S.A. 73: 742-746; P. Tattersall and D.C. Ward (1976), "Rolling hairpin model for the replication of parvovirus and linear chromosomal DNA", Nature 263: 106-109).

However, by means of the formerly known plasmids or nucleic acid constructs it is not possible to appropriately introduce nucleic acids into cells or cell organelles via the protein import route. But this is e.g. a precondition for treating genetically changes of the mitochondrial genomes of patients suffering from neuromuscular and neurodegenerative diseases or carrying out an appropriate mutagenesis in mitochondria or other cell organelles.

Therefore, it was the object of the present invention to develop a construct on a nucleic acid basis which permits the appropriate introduction of nucleic acids into cell and compartments of eukaryotic cells. Furthermore, a process is to be provided of how this construct can reach cell compartments or cells. In addition, the introduced nucleic acid should be such that it can also be incorporated as replicative nucleic acid via cellular protein import routes. Besides properties should be present which result in a controlled transcription and/or replication in cells and in defined aimed compartments of cell, respectively. The process is to be used for the therapy of genetic diseases (changes of the mitochondrial genome) and for the appropriate mutagenesis in eukaryotic and prokaryotic cells. The invention is to meet the following demands:

- universal applicability
- cell-specific, compartment-specific and membrane-specific introduction behavior

- high degree of effectiveness
- low immunogenicity
- minimization of the infection risk
- the introduced nucleic acid (plasmid molecule) is to be replicatable
- the introduced nucleic acid (plasmid molecule) is to be transcribable
- the introduced nucleic acid (plasmid molecule) shall be resistant to nucleases
- the structure of the introduced nucleic acid (plasmid molecule) should be universally usable.

This problem is solved by the features of claims 1), 25), 54), 56), 58), 60) and 61). Advantageous embodiments follow from the subclaims.

In order to be able to appropriately carry a protein within a cell from the site of formation to another compartment or another cell organelle (e.g. the site of action), the protein is usually synthesized as a preprotein (R. Zimmermann et al. (1983), "Biosynthesis and assembly of nuclear-coded mitochondrial membrane proteins in Neurospora crassa", Methods Enzymol. 97: 275-286). In addition to the matured amino acid sequence, the preprotein has what is called a signal sequence. This signal sequence is specific to the aimed compartment and enables that the preprotein can be recognized by surface receptors. The natural obstacle 'membrane' is then overcome by translocating the preprotein through the membrane by an active (several 'transport proteins' are involved in this process) or passive process (direct passage without involvement of further proteins). Thereafter, the signal sequence is usually separated on the site of action by a specific peptidase unless it is a constituent of the matured protein. The matured protein can now unfold its enzymatic activity.

The inventors have recognized that this mechanism can be utilized to appropriately transport nucleic acids across

membranes. In this case, the nucleic acid is not subject to a restriction, i.e. it is possible to use every nucleic acid desired and known, respectively. For this purpose, a cell-specific, compartment-specific or membrane-specific signal sequence is linked with the desired nucleic acid, resulting in a chimerical peptide-nucleic acid fragment. In this context, it is known that the linkage between a nucleic acid and a peptide may occur via the α-amino group of a synthetic KDEL peptide, modified by ε-maleimidocapronic acid-N-hydroxysuccinimide ester (K. Arar *et al.* (1993), "Synthesis of oligonucleotide-peptide conjugates containing a KDEL signal sequence", Tetrahedron Lett. 34: 8087-8090). However, this linkage strategy is completely unusable for the nucleic acid introduction into cell organelles and cells, since here the translocation should occur in analogy to the natural protein transport. Such a transport cannot be expected by blocking the α-amino group of a synthetic peptide by means of a nucleic acid. Therefore, the inventors chose linkage via a carboxy-terminal amino acid. On the one hand, this ensures a 'linear' linkage, on the other hand, the free amino-terminal end of the signal peptide is thus available for the essential steps of the import reaction.

In order to be able to utilize the described transport mechanism also for the introduction of replicative and transcription-active nucleic acids, the nucleic acid is preferably integrated via a homologous recombination into an existing genome or is itself the carrier of the genetic elements, which ensures an autonomous initiation of replication and transcription. Only the latter variant complies with the criterion of universal applicability, since a recombination into an existing cellular genome is successful only under certain conditions and in select cells.

In this case, the use of cyclic DNA represents one possibility, since the DNA polymerases at the end of the new synthesis of the daughter strands return to the initial

point thus guaranteeing a complete DNA replication. Although the use of a double-stranded cyclic plasmid meets all physical criteria for a successful replication in every aimed compartment of the cell, this physical DNA form is confronted with the import pore size which is decisively involved in the appropriate translocation: Even the compact diameter of a superhelical plasmid can be compared with that of globular proteins, therefore, a translocation through a membrane system via the protein import route appears impossible. Here, an approach to a solution consists in the use of linear-cyclic DNA molecules having modified (cyclic) ends but only the diameter of linear DNA molecules. On the one hand, they are no obstacle for the import pore size; on the other hand, these linear-cyclic DNA molecules include all physical preconditions to be able to form replicative and transcription-active plasmids in the mitochondria.

The following is preferably required for the construction of the chimerical peptide-nucleic acid fragment according to the invention as well as for the construction of a replicative and transcription-active nucleic acid portion (plasmid):

- signal peptide and signal sequence, respectively, (cell-specific, compartment-specific, or membrane-specific)
- linkage agent
- nucleic acid (oligonucleotide) which may preferably comprise the following further information:
  - information on the initiation and regulation of transcription and replication,
  - information as to the termination of transcription and replication,
  - multiple cloning site for a nucleic acid to be introduced (to be expressed) additionally,

- possible modifications, so that 'hairpin loops' can be added (cyclization of the ends) which permit linkage with the signal peptide.

The selection of the signal sequence depends on the membrane and membrane system, respectively, which is to be overcome and the aimed compartment of the cell (cell nucleus, mitochondrion, chloroplast) or the cell organelle which is to be obtained. Proteins which are to be introduced e.g. into one of the four mitochondrial compartments (outer mitochondrial membrane, intermembraneous space, inner mitochondrial membrane, matrix space), have compartment-specific signal sequences. In general, signal sequences are chosen for the introduction of nucleic acids which contain a cell-specific, compartment-specific or membrane-specific recognition signal thus directing the attached nucleic acid to its site of action (e.g. inner side of the inner mitochondrial membrane or matrix space). A selection can be made among signal sequences which can transport proteins in the presence or absence of a membrane potential. For the nucleic acid introduction, signal sequences which function irrespective of the membrane potential are preferred, e.g. the signal sequence of ornithine transcarbamylase (OTC) for the transport into the matrix space of the mitochondria (A.L. Horwich *et al.* (1983), "Molecular cloning of the cDNA coding for rat ornithine transcarbamylase", Proc. Natl. Acad. Sci. U.S.A. 80: 4258-4262; J.P. Kraus *et al.* (1985), "A cDNA clone for the precursor of rat mitochondrial ornithine transcarbamylase: comparison of rat and human leader sequences and conservation of catalytic sites", Nucleic. Acids. Res. 13: 943-952). Basically, the pure signal sequence suffices for the transport into the aimed compartment. However, preferable is to select signal sequences which additionally have a cell-specific or compartment-specific peptidase cleavage site. In the most favorable case, this 'cleavage site' is within the signal sequence but can also be attached thereto by additional

amino acids to ensure the cleavage of the signal sequence when the aimed compartment has been reached (e.g. the signal sequence of human OTC can be prolonged by ten additional amino acids of the matured OTC). This ensures that the nucleic acid can be separated from the signal peptide in the aimed compartment, so that the action of the nucleic acid fully unfolds. The selected signal sequence is prepared biologically (purification of natural signal sequences or cloning and expression of the signal sequence in a eukaryotic or prokaryotic expression system) but preferably in a chemical-synthetic way.

In order to ensure a linear chemical linkage between nucleic acid and signal peptide, the signal peptide is linked via a linkage agent which is generally linked therewith via amino acids, preferably via amino acids having reactive side groups, preferably via an individual cysteine or lysine at the carboxy-terminal end of the signal peptide. A bifunctional cross-linker serves as a linkage reagent, preferably a heterobifunctional cross-linker which has a second reactive group, preferably an aminoreactive group, in addition to a thiol-reactive group at the signal peptide when a cysteine is used as the linkage site (e.g. m-maleinimidobenzoyl-N-hydroxy-succinimide ester, MBS and its derivatives).

The nucleic acid also has a linkage site which should be compatible with the selected cross-linker. When MBS is used, the oligonucleotide should have an amino function or thiol function. The linkage group of the nucleic acid can be introduced via the chemical synthesis of the oligonucleotide and is generally localized at the 5' end, at the 3' end, but preferably directly at a modified base, e.g. as 5' amino linker (TFA amino linker Amidite^{R}, 1,6-(N-trifluoroacetylamino)-hexyl-ß-cyanoethyl-N,N-diisopropyl phosphoramidite, Pharmacia) or a 5' thiol linker (THIOL-C6 Phosphoramidit^{R}, MWG Biotech) at a free 5' hydroxy/phosphate group, as 3' amino linker (3' aminomodifier-C7-CPG-Synthesesäulen^{R}, MWG Biotech) at a

free 3' hydroxy/phosphate group, but preferably as amino-modified base analog, preferably amino-modified deoxyuridine (Amino-Modifier-dT^{R}, 5'-dimethoxy-trityl-5[N-(trifluoroacetylaminohexyl)-3-acrylimido]-2'-deoxyuridine, 3'-[2-cyanoethyl)-(N,N-diisopropyl)]phosphoramidite, Glen Research) within the sequence. In this case, the reactive group compatible with the cross-linker used is spaced from the 5' end or 3' end of the oligonucleotide or the modified base by at least one C2-spacer unit, but preferably by a C6-spacer unit. The nucleic acid (oligonucleotide) including a reactive linkage group then comprises at least two nucleotides.

In order to increase the stability of the nucleic acid (oligonucleotide) over cellular and extracellular nucleases, the chemically synthesized nucleic acids can be protected by a sulfurizing reagent (Beaucage-Reagenz^{R}, MWG-Biotech). The phosphorus diester bonds of the nucleic acid are converted into phosphorus thioate bonds in the chemical synthesis. This oligonucleotide can then be used for the enzymatic amplification of nucleic acids, extended by further linkage reactions with other nucleic acids or used directly.

In order to directly use the chimerical peptide nucleic acid fragment, the nucleic acid (oligonucleotide) should have a secondary structure that can be hybridized, preferably without internal homologies so as to be able to form a linear single-strand structure. This ensures that the nucleic acid (oligonucleotide) of the chimerical peptide-nucleic acid fragment can unfold a biochemical/therapeutic effect without further nucleic acid linkages.

However, for linkage with the signal sequence it is preferred to use nucleic acids (oligonucleotides) which have two further properties:

1. The sequence is preferably partially palindromic, has a blunt 5'-3' end ('blunt end'), an overhanging 3' end ('sticky end'), but has especially an overhanging, phosphorylated 5' end ('sticky end'), especially preferably an overhanging 5' end which comprises 4 nucleotides and has no self-homology (palindromic sequence). As a result, a stable, monomeric secondary structure ('hairpin loop') may form. The overhanging 5' end serves for linking defined nucleic acids, antisense oligonucleotides, but preferably transcribable and replicatable genes.

2. In the apex of the 'loop', the oligonucleotide carries a modified base which carries a grouping reactive with respect to the cross-linker, preferably an amino-modified 2'-deoxythymidine. In this case, the amino function of this modified base enables the linkage reaction between MBS and oligonucleotide.

The chimerical peptide-nucleic acid fragment is suitable for appropriately introducing nucleic acids into cells and cell organelles (e.g. nucleus, chloroplast), particularly for introducing ribonucleic acids (mRNA, 'antisense' oligonucleotides) and deoxyribonucleic acids (complete gene, 'antisense' oligonucleotides). It is especially suitable for the introduction of transcribable and processable genes into mitochondria, but even more suitable for the introduction of replicative, transcription-active and processable linear-cyclic nucleic acids (plasmids).

In a preferred embodiment, a transcribable gene is linked to the nucleic acid, containing the reactive linkage site, or to the chimerical peptide-nucleic acid fragment. This is effected preferably by the amplification of a gene, preferably a cloned gene consisting of a mitochondrial promoter, preferably the promoter of the light DNA strand (O_L, nt 490 - nt 369) and the gene to be expressed in a processable form, preferably a mitochondrial gene, preferably a mitochondrial transfer RNA, preferably the

mitochondrial $tRNA^{Leu(UUR)}$ (nt 3204 - nt 3345) (S. Anderson *et al.* (1981), "Sequence and organization of the human mitochondrial genome", Nature 290: 457-465). Following the enzymatic amplification of the gene, the linkage to the nucleic acid, containing the reactive linkage site, or to the chimerical peptide-nucleic acid fragment can be effected via a 'blunt end' ligation, but preferably a 'sticky end' ligation. For this purpose, the nucleic acid to be linked has at least one end capable of linkage, which consists preferably of a 5' overhang which comprises 4 nucleotides and has no self-homology (palindromic sequence). If both ends are to be linked with 'hairpin loops', a nucleic acid will preferably be selected which has differing 5' overhangs which comprise 4 nucleotides and have no self-homology. It is especially preferred to use nucleic acids whose 5' ends also have no homology with respect to one another. For the modification of the ends (cyclization) it is then preferred to use two different 'hairpin loops', one being specific (complementary) to the 'left' plasmid end and the other being specific to the 'right' plasmid end of the nucleic acid. In order to increase the stability of the nucleic acid over cellular and extracellular nucleases, the phosphorus diester bonds of the nucleic acid can be substituted with phosphorus thioate bonds and thus be protected if modified phosphorus thioate nucleotides have been used already in the enzymatic amplification.

A process comprising the following steps is suitable for the production of a chimerical peptide-nucleic acid fragment:

(a) Reaction of a nucleic acid (oligonucleotide), containing a functional linkage group, with a linkage agent.

(b) Reaction of the construct resulting from (a) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL signal sequence, and

(c) optional extension of the chimerical peptide-nucleic acid fragment resulting from (b) by further DNA or RNA fragments.

In another preferred embodiment, the chimerical peptide-nucleic acid fragment can be produced by the following steps:

(a) Optional extension of the nucleic acid, containing a functional linkage group, by further DNA or RNA fragments.
(b) Reaction of the nucleic acid with functional linkage group or the extended nucleic acid resulting from (a) with a linkage agent.
(c) Reaction of the construct resulting from (b) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL sequence.

In another embodiment which is a linear-cyclic nucleic acid in the form of a plasmid, the selection of the nucleic acid depends on the genetic information which shall be expressed in which cell and in which aimed compartment of the cell. In this connection, nucleic acids which are to be transcribed have to have a suitable promoter. For example, if a gene is to be expressed in the mitochondrial matrix, a mitochondrial promoter can be chosen, preferably the promoter of the light mtDNA strand. The transcription is controlled in other cell compartments (e.g. nucleus, chloroplast) by compartment-specific promoters.

The transcription is usually regulated by what is called transcription regulation sequences, preferably mitochondrial transcription regulation sequences. In general, these sequences comprise at least binding sites for factors which initiate the transcription (transcription initiation factor) as well as the binding site for the RNA synthesis apparatus. If a transcription is to be initiated in the mitochondria, binding sequences of the mitochondrial

transcription factors and of the RNA polymerase, particularly of the mitochondrial transcription factor 1 and the mitochondrial RNA polymerase, will be suitable. In other cell compartments (e.g. nucleus, chloroplast), the transcription can be controlled by compartment-specific transcription-regulation sequences.

In order to be able to regulate the transcription, the plasmid has transcription regulation sequences which are attached preferably in the 3' direction of the transcription initiation site (promoter). For example, if the transcription of a mitochondrial transformation plasmid is to be regulated, the control elements will be suitable for the H-strand and L-strand transcription of the mitochondrial genome, however preferable would be the so-called 'conserved sequence blocks' which terminate the transcription of the L-strand and simultaneously enable the transition to the DNA replication. In order to induce the exclusive transcription of the desired gene (optionally the desired genes in a polycistronic transcription), the transcription is discontinued on a suitable site behind the 3' end of the expressive gene / genes. This is achieved by the insertion of a suitable transcription-termination site, preferably arranged in the 3' direction to the promoter. For the regulated expression, the binding sequence for a bidirectionally acting transcription-termination factor is especially suitable in this case. For the transcription-termination in the mitochondria, a binding motif of a mitochondrial transcription-termination factor is preferably chosen here. At the same time, the formation of 'antisense RNA' of the head-to-head-linked dimeric plasmids is suppressed by the use of the transcription-termination factor binding sequence.

The selection of transformed cells can be controlled via the expression of a reporter gene. Expressive genes whose expression result in a macroscopic change of the phenotype are especially suitable as reporter or selection gene. A selection is made among genes which produce resistances to

antibiotics, for example. In particular, the resistance genes for oligomycin (OLI) or chloramphenicol (CAP) are suitable for the use in a mitochondrial transformation system. In this connection, the mitochondrial chloramphenicol resistance gene appears to be a particularly suitable selection gene, since CAP-sensitive cell lines already change their phenotype at a portion of about 10 % of the 16 S $rRNA^{CAP+}$ gene.

The replication of the nucleic acid can be realized by an initiation site for the DNA replication (replication origin). Therefore, the chimerical peptide-nucleic acid fragment in the form of a plasmid has to have at least one replication origin. In this connection, the orientation of the replication origin can be arranged irrespective of the expressive gene (genes), but preferably the replication origin is arranged in the 3' direction of the promoter. A suitable replication origin for a mitochondrial transformation plasmid would be a mitochondrial replication origin. In particular, the origin of replication of the heavy mtDNA strand is suitable in this case. It preferably has at least one 'conserved sequence block'. The replication can be controlled via what is called regulation sequences for the replication. For this purpose, the plasmid has to have at least one such sequence motif which is preferably arranged in the 3' direction of the promoter and the replication origin. If the replication in the mitochondria is to be regulated, a mitochondrial replication regulation sequence will be especially suitable. It is preferred to use a motif which comprises at least one of the 'termination associated sequences'. In other cell compartments (e.g. nucleus, chloroplast), the replication is initiated at least via one compartment-specific replication origin and controlled via compartment-specific replication regulation sequences.

In order to permit cloning of different genes into the plasmid molecule, the plasmid nucleic acid also has to have a suitable cloning module (multiple cloning site) which has

the most widely differing recognition sequences for restriction endonucleases. Here, rare recognition sequences which do not occur on other sites of the plasmid are especially suitable. The cloning module can be incorporated into any site of the transformation plasmid. If the region of the cloning site is to be integrated into the transcription of the selection gene, the insertion of the multiple cloning site in the 3' direction of the promoter and in the 5' direction of the transcription termination site will be suitable. The integration of the multiple cloning site in the 5' direction of the selection gene is especially suitable, since in this case the use of the selection system is simultaneously accompanied by a transcription of the region of the multiple cloning site.

In order to permit the autonomous replication in every aimed compartment of a cell when a nucleic acid is used, it has to be ensured that, after the synthesis of the daughter strand, the DNA replication enzymes return to the synthesis starting point again to guarantee the covalent linkage of the 3' end with the 5' end of the newly synthesized daughter strand by corresponding enzymes. For this purpose, a linear nucleic acid plasmid is suitable which can be converted into a cyclic nucleic acid. The plasmid ends can be cyclized via the use of what is called ligation-capable (phosphorylated) ends of nucleic acid. For this purpose, the use of a 'blunt end' nucleic acid or a nucleic having a overhanging 3' ends, but preferably a nucleic acid having overhanging 5' ends is particularly suitable. In this case, the overhanging ends should comprise at least one nucleotide. However, it is preferred to use overhanging 5' ends which are formed of four nucleotides. They have preferably no self-homology (palindromic sequence) and are also preferably not complementary to one another in order to suppress the formation of dimers in a subsequent nucleic acid linkage.

The cyclization of the prepared plasmid ends is arranged by synthetic oligonucleotides. They have a partial self-

homology (partially palindromic sequence) and are thus capable to form what is called 'hairpin loop' structures. The partially palindromic sequence results in the formation of a stable, preferably monomeric secondary structure ('hairpin loop') having a blunt 5'-3' end (blunt end), an overhanging 3' end ('sticky end'), but preferably an overhanging 5' end. These oligonucleotides are especially preferred when they have a phosphorylated 5' end. When synthetic oligonucleotides having 'hairpin loop' structure are used, the linear plasmid DNA can be converted into a linear-cyclic system. The ends of the two oligonucleotides are preferably complementary to one end of the prepared plasmid nucleic acid each. For this purpose, two different 'hairpin loops' are preferably used, one being specific (complementary) to the 'left' plasmid end, one being specific (complementary) to the 'right' plasmid end to suppress the dimer formation. At least one of the two 'hairpin loop' oligonucleotides may have at least one modified nucleotide. It guarantees the linkage site to a signal peptide, so that the nucleic acid transport can be arranged via the protein import route. In the model case, this linkage site (modified nucleotide) is placed at one of the unpaired positions of the 'loop'. A chemically reactive group, particularly an amino or thiol function, is especially suitable as linkage site.

In order to prepare the ends of the transformation plasmid for the modification (cyclization), it has to be ensured that the plasmid ends are complementary to the ends of the oligonucleotides ('hairpin loops'). On the one hand, this succeeds by amplifying the plasmid DNA with suitable oligonucleotides which have at least one recognition sequence for a restriction endonuclease. In this case, recognition sequences for restriction endonucleases are suitable which do not occur repeatedly in the plasmid sequence. Especially suitable is the use of recognition sequences for restriction endonucleases generating overhanging ends ('sticky ends'), particularly those which produce overhanging 5' ends, preferably outside the own

recognition sequence. In this connection, the recognition sequence for the restriction endonuclease *Bsa* I ($GGTCTCN_1N_5$) is especially suitable. On the other hand, the use of a cloned nucleic acid which already has the recognition sequences for a restriction endonuclease, preferably *Bsa* I, is suitable. As a result, the enzymatic amplification can be omitted and the nucleic acid obtained by plasmid preparation/restriction enzyme treatment can be used directly. It is preferred that the cloned nucleic acid already includes the recognition sequence for the restriction endonuclease *Bsa* I at both ends.

Various methods are available for purifying the transformation plasmid. Here, the main objective is to separate the cyclic plasmid molecule from the unreacted educts. The use of DNA-degrading enzymes are proved to be suitable in this connection. In particular, it is recommended to use enzymes which have a 5'-3' or 3'-5' exonuclease activity. Particularly the use of the exonuclease III leads to the complete hydrolysis of unreacted educts while the cyclic plasmid DNA remains intact (no free 5' ends or 3' ends). The reaction products can be purified either via electrophoretic or chromatographic processes but also by precipitation. A selection can be made among different purification processes. On the one hand, the cyclic nucleic acid conjugated with the linkage agent and the signal peptide can be treated with an exonuclease, preferably exonuclease III, and then be purified via chromatographic, electrophoretic purification and precipitation, respectively. On the other hand, the cyclic plasmid DNA can also be treated with an exonuclease, preferably exonuclease III, be purified and subsequently be conjugated with the linkage agent and the signal peptide and be purified via a chromatographic, electrophoretic purification and precipitation, respectively.

The linkage with a signal peptide can be realized by means of modified oligonucleotides. This peptide directs *in vivo*

the transformation plasmid into the desired cell compartment. To this end, either the transformation plasmid can first be reacted with the modified oligonucleotide (ligation) and then the conjugation with the linkage agent and the signal peptide can take place or the modified oligonucleotide is first conjugated with the linkage agent and the signal peptide and then be used for the cyclizing the transformation plasmid ends (ligation).

The transformation system (cellular transformation) can overcome the cell membrane by various methods. Here, 'particle gun' system or microinjection are suitable, but electroporation and lipotransfection are preferred. All methods ensure the introduction of the linear-cyclic peptide nucleic acid plasmid into the cytosol of the cell from where the plasmid is directed to its site of action (aimed compartment) by the conjugated signal peptide.

As compared to the prior art transformation and infection methods, mentioned in the introductory part of the description, this process offers, for the first time, the possibility of appropriately introducing nucleic acids into cells and cell organelles. The selection of the signal sequence can determined the aimed compartment which is to be reached in this case (cytosol, nucleus, mitochondrion, chloroplast, etc.). Along with the compartment-specific and cell-specific introduction behavior, this process distinguishes itself by its universal applicability. Both prokaryotic and eukaryotic cells and cell systems can be treated with the translocation vector. Since a natural transport system of the membranes is used for the appropriate introduction, the treatment of the cells or cell organelles with membrane-permeabilizing agents becomes superfluous (e.g. calcium chloride method, see above).

When a replicative and transcription-active nucleic acid is used, the plasmid does not unfold its full size until the first replication cycle has been completed: As a genuine cyclic plasmid (artificial chromosome) it now has the

double genetic information (head-to-head linked plasmid dimers). In particular with respect to the use of this system for a somatic gene therapy, this behaviour is induced intentionally and of decisive importance, since the genes to be expressed have to compete with the defect genes of the cells. In addition to this highest possible effectiveness, the system distinguishes itself through the fact that it does not have to be integrated into a genome via a recombination step, such as retroviral systems, so as to become replicative. As a result, uncontrollable side-effects (undesired recombination) are already suppressed to the highest possible degree from the start. Therefore, the application of this plasmid system can be expected without great safety risk.

The present invention is explained particularly by the figures, wherein:

Fig. 1 shows a signal peptide of the ornithine transcarbamylase of rats as well as a DNA sequence suitable for the introduction. Top: signal peptide of the ornithine transcarbamylase of rats (32 amino acids), extended by 10 N-terminal amino acids of the matured protein and an additional cysteine as linkage site. The peptide sequence is shown in the international one-letter code; middle: a partially palindromic DNA sequence suitable for the introduction and consisting of 39 nucleotides having an amino-modified T at nucleotide position 22; bottom: marked secondary structure of the oligonucleotide having an overhanging 5' end and a modified nucleotide in the vertex of the 'loop'.

Fig. 2 shows the structure of the amino-modified 2'-deoxythymidine, R: nucleic acid residues.

Fig. 3 shows a diagram of the chimerical peptide-nucleic acid fragment, consisting of amino-modified

oligonucleotide (39 nucleotides) with marked 'hairpin loop', cross-linker and signal peptide. CL: cross-linker.

Fig. 4 shows the electrophoretic separation of the linkage product resulting from amino-modified oligonucleotide (39 nucleotides), m-maleimidobenzoyl-N-hydroxy-succinimide ester (MBS) and signal peptide of the ornithine transcarbamylase of rats (42 amino acids, extended by a cysteine at the C terminus).

Fig. 5a shows a flow diagram of the peptide-DNA fusion, cloning, amplification and linkage of the transcribable and processable mitochondrial tRNA gene to be introduced (S. Anderson et al. (1981), "Sequence and organization of the human mitochondrial genome", Nature 290: 457-465). CL: cross-linker (MBS); MCS: multiple cloning site of pBluescript^{R} (Stratagene), mtTF: binding site of the mitochondrial transcription factor; RNA-Pol: binding site of the mitochondrial RNA polymerase; tRNA Leucin: gene of the mitochondrial transfer RNA for leucine (UUR); *Sac II, Apa I, Eco RI*: sites for restriction endonucleases; the cloned mitochondrial sequences were numbered in accordance with the published sequence of the human mitochondrial genome (S. Anderson *et al.* (1981), "Sequence and organization of the human mitochondrial genome", Nature 290: 457-465).

Fig. 5b shows the sequence of the cloned $tRNA^{Leu(UUR)}$ gene.

Fig. 6a/b shows a presentation of the ^{32}P radiation of the DNA as well as the enzyme activities for adenylate kinase, cytochrome c oxidase and malate dehydrogenase (y axes) in 11 fractions (x axes) of a mitochondria-sucrose gradient density

centrifugation. The portion of the particular radiation/enzyme activity, expressed as percentage of the total radiation/enzyme activity which was plotted against the gradient is illustrated. ADK: adenylate kinase; COX: cytochrome c oxidase; MDH: malate dehydrogenase.

Fig. 7a/b shows a presentation of the ^{32}P radiation of the DNA as well as the enzyme activities for adenylate kinase, cytochrome c oxidase and malate dehydrogenase (y axes) in 11 fractions (x axes) of a mitoplast-sucrose gradient density centrifugation. The portion of the particular radiation/enzyme activity, expressed as percentage of the total radiation/enzyme activity which was plotted against the gradient, is illustrated. ADK: adenylate kinase; COX: cytochrome c oxidase; MDH: malate dehydrogenase.

Fig. 8 shows the cloning of the nucleic acid portion of the peptide-nucleic acid plasmid into pBluescript (plasmid 1). Using the two oligonucleotides (primers 1 and 2), the gene section of nucleotide 15903 to nucleotide 677 was amplified enzymatically from mitochondrial HeLa DNA (comprises: promoter characterized by the binding sites for the mitochondrial transcription factors and the RNA polymerase; replication origin characterized by what is called 'conserved sequence blocks'; regulation of the DNA replication characterized by the 'TAS' motifs). Since the oligonucleotides contain recognition sequences for the restriction endonucleases *Xho* I and *Pst* I, the ends of the amplified nucleic acid can be modified such that they are compatible with a vector arm of pBluescript, on the one hand, and compatible with the hybrid of the oligonucleotides MCS/TTS 1 and 2, on the other hand. In addition to a multiple cloning site,

they also comprise a transcription termination sequence which is responsible for the regulated transcription. The ligation product is then transformed into *E. coli* XL 1. Following the plasmid isolation of insert-carrying *E. coli* colonies, the nucleic acids were subjected to RFLP and sequence analysis.

Fig. 9 shows the sequence of the oligonucleotides MCS/TTS 1 and 2. The oligonucleotides MCS 1 and 2 were prepared synthetically and comprise recognition sequences for nine different restriction endonucleases as well as a sequence motif which can suppress the transcription bidirectionally. The oligonucleotides are complementary and can thus form a hybrid. The overhanging ends are part of the recognition sequences for the restriction endonucleases *Pst* I and *Bam* HI.

Fig. 10 shows the nucleotide sequence of the nucleic acid portion of the peptide-nucleic acid plasmid (plasmid 1).

Fig. 11 shows the cloning of the reporter gene into the nucleic acid portion of the peptide-nucleic acid plasmid into pBluescript (plasmid 2). Using the two oligonucleotides (primers 3 and 4), the gene section of nucleotide 1562 to nucleotide 3359 was amplified enzymatically from a DNA extract of a human CAP-resistant cell line (comprises: part of the 12 S rRNA gene, $tRNA^{Val}$ gene, 16 S $rRNA^{CAP+}$ gene, $tRNA^{Leu}$ gene, part of ND 1 gene). Since the oligonucleotides contain recognition sequences for the restriction endonucleases *Hind* III and *Bcl* I, the ends of the amplified nucleic acid can be modified such that they are compatible with the multiple cloning site (MCS) of the peptide-nucleic acid plasmid (plasmid 1). The ligation

product is then transformed in *E. coli* XL 1 Blue. Following the plasmid isolation of insert-carrying *E. coli* colonies, the nucleic acids were subjected to RFLP and sequence analysis and are available for the described experiments.

Fig. 12 shows the nucleotide sequence of the nucleic acid portion of the peptide-nucleic acid plasmid including the reporter gene (plasmid 2).

Fig. 13a shows the reaction run of the cyclization of the nucleic acid portion as well as the conjugation of the nucleic acid portion with a signal peptide. The nucleic acid portion of the peptide-nucleic acid plasmid can be obtained via a plasmid preparation or an enzymatic amplification. In both cases, the treatment with the restriction endonuclease *Bsa* I results in an intermediate product capable of ligation. It can be reacted directly with the monomerized 'hairpin loops'. The reaction product is freed by an exonuclease III treatment from non-specific (non-cyclic) reaction products and educts, is purified and conjugated with the signal peptide via a cross-linker. As an alternative, one of the two 'hairpin loops' can first be conjugated with the signal peptide via a cross-linker before the cyclizing ligation reaction is carried out. A purification of the reaction product follows an exonuclease III treatment here as well.

Fig. 13b shows the structure and sequence of the 'hairpin loop' oligonucleotides HP 1 and 2.

Fig. 14 shows the monomerization of a 'hairpin loop' oligonucleotide. The synthetic 'hairpin loops' HP 1 and 2 can be monomerized by a thermal or alkaline denaturation. This figure shows a standard agarose gel: lane 1, molecular weight

standard (ΦX 174 RF DNA treated with the restriction endonuclease *Hae* III), lane 2: HP 1, synthesis product; lane 3: HP 1, thermally monomerized.

Fig. 15a shows a ligation reaction between the nucleic acid portion of the peptide-nucleic acid plasmid (plasmid 2) and the 'hairpin loops' HP 1 and 2. This figure shows a standard agarose gel: lane 1, cloned nucleic acid portion of the peptide-nucleic acid portion in pBluescript treated with the restriction endonuclease *Bsa* I, lane 2: ligation of the reaction products resulting from lane 1 with the 'hairpin loops' HP 1 and 2; lane 3, treatment of the reaction products resulting from lane 2 with exonuclease III; lane 4, molecular weight standard (λDNA treated with the restriction endonucleases *Hind* III and *Eco* RI).

Fig. 15b shows the examination of the purified ligation product by a *Mae* III-RFLP analysis. This figure illustrates a standard agarose gel: lane 1, enzymatically amplified nucleic acid portion following a *Mae III* treatment; lane 2: purified ligation product of the enzymatically amplified nucleic acid portion following a *Mae* III treatment; lane 3: purified product of the plasmid DNA ligation following a *Mae* III treatment; lane 4, molecular weight standard (ΦX 174 RF DNA treated with the restriction endonuclease *Hae* III).

Fig. 16 shows the transcription and replication of the peptide-nucleic acid plasmid. This figure illustrates a standard agarose gel: lane 1, molecular weight standard (λDNA treated with the restriction endonucleases *Hind* III and *Eco* RI); lane 2, untreated peptide-nucleic acid plasmid; lane 3: *in vitro*-obtained transcription products

of the peptide-nucleic acid plasmid; lane 4: *in vitro*-obtained replication and transcription products of the peptide-nucleic acid plasmid; lane 5, *in vivo*-obtained replication and transcription products of the peptide nucleic acid plasmid; lane 6, untreated peptide-nucleic acid plasmid.

The present invention is now explained by way of the below examples which, however, shall not at all restrict the invention.

Example 1:
Introduction of a chimerical peptide-nucleic acid fragment into the mitochondria

The overcoming of the mitochondrial double membrane system with a DNA translocation vector was studied to prove that nucleic acids can be transported appropriately across membranes by the above-described process. For this purpose, the mitochondrial signal sequence of the ornithine transcarbamylase (A.L. Horwich *et al.* (1983), "Molecular cloning of the cDNA coding for rat ornithine transcarbamylase", Proc. Natl. Aca. Sci. U.S.A. 80: 4258-4262) (enzyme of urea cycle, naturally localized in the matrix of the mitochondria) was chemically prepared and purified. The original sequence was extended by a cysteine at the C terminus as reactive group for the subsequent linkage with the DNA (see fig. 1). This ensured that the heterobifunctional cross-linker (MBS) can only be linked with the thiol group of the only cysteine. A DNA oligonucleotide (39 nucleotides) were chosen as linkage partner. It distinguishes itself by two special features:

1. The sequence is partially palindromic and has an overhanging, phosphorylated 5' end (see fig. 1). As a result, what is called a 'hairpin loop' can form. The overhanging 5' end serves for ligating to this

oligonucleotide defined nucleic acids which can then be imported into the mitochondria.

2. The oligonucleotide carries a modified base in the vertex of the 'loop' (see fig. 1). In this case, an amino-modified 2'-deoxythymidine is concerned (see fig. 2). Here, the amino function of the modified bases in this connection enables the linkage reaction between MBS and oligonucleotide.

The three reaction partners (oligonucleotide, MBS and peptide) are linked in individual reaction steps. Firstly, the oligonucleotide (50 pmoles) is reacted in a buffer (100 µl; 50 mM potassium phosphate, pH 7.6) with MBS (10 nmoles dissolved in DMSO) (reaction time: 60 min.; reaction temperature: 20°C). Unreacted MBS is separated via a Nick-spin column^{R}, (Sephadex G 50, Pharmacia) which was equilibrated with 50 mM of potassium phosphate (pH 6.0). The eluate contains the desired reaction product and is reacted in another reaction step with the peptide (2.5 nmoles) (reaction time: 60 min.; reaction temperature 20°C). The reaction was stopped by the addition of dithiothreitol (2 mM). The linkage product (chimera, see fig. 3) was separated via a preparative gel electrophoresis of unreacted educts and isolated from the gel by electroelution (see fig. 4). Differing nucleic acids can now be linked by simple ligation to the overhanging 5' end of the oligonucleotide.

A 283 bp long double-stranded DNA (dsDNA) was amplified via an enzymatic reaction (PCR) in the below experiment. For this purpose, a DNA fragment cloned into pBluescript^{R} (Stratagene) served as template DNA, which fragment in addition to the human mitochondrial promoter of the light strand (P_L, nt 902 - nt 369) included the gene for the mitochondrial transfer RNA leucine ($tRNA^{Leuc(UUR)}$, nt 3204 - nt 4126) (see fig. 5). Two oligonucleotides served as amplification primers, primer 1 having a non-complementary 5' end (see fig. 5). The dsDNA was modified by the 3'-5'

exonuclease activity of the T4 DNA polymerase (incubation in the presence of 1 mM dGTP) which can produce overhanging 5' ends under conditions with which a person skilled in the art is familiar (C. Aslanidis *et al.* (1990), "Ligation-independent cloning of PCR products (LIC-PCR)", Nucleic. Acids. Res. 18: 6069-6074).

Together with the previously conjugated peptide-MBS oligonucleotide the PCR-amplified DNA could be joined using the T4 DNA ligase. In order to be able to easily detect the linkage partners after the introduction into the mitochondria, the free 5'-OH group of the ligated DNA was phosphorylated radioactively by an enzymatic reaction (A. Novogrodsky *et al.* (1966), "The enzymatic phosphorylation of ribonucleic acid and deoxyribonucleic acid, I. Phosphorylation at 5'-hydroxyl termini", J. Biol. Chem. 241: 2923-2932; A. Novogrodsky *et al.* (1966), "The enzymatic phosphorylation of ribonucleic acid and deoxyribonucleic acid. II. Further properties of the 5'-hydroxyl polynucleotide kinase", J. Biol. Chem. 241: 2933-2943).

A fresh rat liver was comminuted for the isolation of mitochondria, suspended in 25 mM HEPES, 250 mM saccharose, 2 mM EDTA, 52 μM BSA and homogenized in a glass homogenizer (50 ml). Cell membranes, cellular debris and nuclei were centrifuged off at 3000 g and the supernatant was prepared for another centrifugation. For this purpose, the supernatant was placed in cooled centrifuge cups and centrifuged at 8000 g. The isolated mitochondria were resuspended in 200 ml of the same buffer and centrifuged again at 8000 g. The purified mitochondria pellet was resuspended in an equal volume of the same buffer and energized by the addition of 25 mM succinate, 25 mM pyruvate and 15 mM malate. The protein content of the suspension was determined by a Bradford Testkit^{R} (Pierce). 200 μg of mitochondrial protein (energized mitochondria) were incubated together with 10 pmoles of the chimera at 37°C for 60 min. (0.6 M sorbitol, 10 mM potassium phosphate

pH 7.4, 1 mM ATP, 2 mM $MgCl_2$, 1 % BSA). The mitochondria were reisolated by centrifugation at 8000 g, resuspended in 0.6 M sorbitol, 10 mM potassium phosphate pH 7.4, 2 mM $MgCl_2$, 1 % BSA, 10 U/ml DNAse I and incubated at 37°C for 30 min. This washing step was repeated twice to remove non-specifically adhering molecules. For proving that the chimera is associated with the mitochondria, the re-isolated mitochondria were purified via sucrose gradient density centrifugation. The individual fractions of the gradient were analyzed to localize the chimera and the mitochondria. The adenylate kinase which determines cytochrome-c oxidate and malate dehydrogenase activity was used as marker for the mitochondria, while the chimera could be identified via the ^{32}P radiation measurement (see fig. 6). An analog experiment for determining the non-specific DNA introduction was carried out with the same DNA which was not linked with the signal peptide (see fig. 6). It was derived from the measurements that 65 % of the chimera used segregated specifically with the mitochondria, whereas the non-specific DNA incorporation was less than 5 % of the DNA used. In order to show that the chimera is not only associated with the surface of the mitochondria (membrane, import receptor), the re-isolated mitochondria were not fractioned into the three compartments of outer mitochondria membrane/intermembranous space, inner mitochondrial membrane and matrix space. For this purpose, the mitochondria were incubated with digitonin (final concentration: 1.2 % w/v digitonin) and the resulting mitoplasts were separated via a sucrose gradient density centrifugation, collected in fractions and the activities of marker enzymes (adenylate kinase: intermembranous space, cytochrome c oxidase: inner mitochondrial membrane; malate dehydrogenase: matrix space) were determined according to Schnaitman and Grennawalt (C. Schnaitman *et al.* (1968), "Enzymatic properties of the inner and outer membranes of rat liver mitochondria", J. Cell Biol. 38: 158-175; C. Schnaitman *et al.* (1967), "The submitochondrial localization of monoamine oxidase. An enzymatic marker for the outer membrane of rat liver mitochondria", J. Cell

Biol. 32: 719-735) (see fig. 7). An analog experiment for determining the non-specific DNA incorporation was carried out with the same DNA which was not linked with the signal peptide (see fig. 7). It was derived from the measurements that 45 % of the chimera are associated with the mitoplasts, whereas the non-specifically adhering DNA could be assessed to be less than 3 %. The isolated mitoplasts (loss of the outer membrane and the intermembranous space) were lyzed by Lubrol^{R} (0.16 mg/mg protein; ICN) and separated into the compartments of inner mitochondrial membrane (pellet) and matrix space (supernatant) by ultracentrifugation at 144,000 g. The compartments were assigned via the measurement of the activities of the cytochrome c oxidase (inner mitochondrial membrane) and the malate dehydrogenase (matrix space). The chimera was measured via the detection of the ^{32}P radiation in the scintillation counter and the result was 75 % segregation with the matrix of the mitochondria, while 25 % of the chimera remained associated with the inner membrane of the mitochondria (incomplete translocation).

Example 2

Incorporation of a replicative and transcription-active chimerical peptide-nucleic acid fragment (plasmid) into the mitochondria of living cells

In order to prove that a linear peptide-nucleic acid plasmid having cyclic ends ('hairpin loops') can overcome membranes *in vivo* via the protein import route and can be transcribed and replicated in spite of the chemical linkage with a signal peptide, the transcription and replication behavior were studied after the transfection of cells and the import into the matrix of the mitochondria. For this purpose, the signal peptide of the mitochondrial ornithine transcarbamylase was prepared synthetically, purified and linked with a nucleic acid plasmid.

Precondition for the examination of the correct transcription and replication behavior is the physical

structure of the plasmid: for the experiment described below, a 3232 bp long double-stranded vector DNA (dsDNA) was cloned into pBluescript^{R} (Stratagene). For this purpose, the region of the mitochondrial genome was amplified via two modified oligonucleotides (primer 1, hybridized with the nucleotides 15903-15924 of the human mtDNA, includes at the 5' end an extension by the sequence TGTAGctgcag for the incorporation of a *Pst* I site; primer 2, hybridized with the nucleotides 677-657 of the human mtDNA, includes at the 5' end an extension by the sequence TTGCATGctcgagGGTCTCAGGG for the incorporation of an *Xho* I site), which comprised the promoter of the light DNA strand, the origin of the mtDNA replication of the heavy strand, the regulation motifs for the transcription (CSBs, 'conserved sequence blocks') as well as the regulation site for the DNA replication ('TAS', termination associated sequences, (D.C. Wallace (1989), "Report of the committee on human mitochondrial DNA", Cytogenet. Cell Genet. 51: 612-621) (see fig. 8). A multiple cloning site was inserted behind this fragment (3' direction), which is to permit an easy linkage with a gene to be expressed. The multiple cloning site (MCS/TTS) was produced via a chemical synthesis of two complementary oligonucleotides (MCS/TTS 1 and 2) which contain the recognition sequences for various restriction endonucleases (see fig. 9). Under conditions with which a person skilled in the art is familiar, the two oligonucleotides form hybrids which, after the phosphorylation with T4 DNA polynucleotide kinase, can be used for the ligation. In this connection, the hybrids distinguish themselves by 5' and 3' single-stranded-overhanging ends which are complementary to a *Pst* I, on the one hand, and are complementary to a *Bam* HI site, on the other hand (see fig. 9). Together with the multiple cloning site, the synthetic oligonucleotides MCS/TTS 1 and 2 also comprise a bidirectional mitochondrial transcription termination sequence (see fig. 9). It is arranged in the 3' direction of the MCS and ensures that the transcription on this site is discontinued thus correctly forming terminated transcripts forming. This sequence motif also ensures that

in the cyclic plasmid system no 'antisense RNA' is expressed. The ligation reaction between pBluescript, PCR-amplified fragment and the MCS/TTS hybrids took place in a stoichiometry of 1:2:2 under conditions with which a person skilled in the art is familiar. After the transformation, several E. coli colonies (clones) could be isolated and characterized. For this purpose, the corresponding plasmid DNA was subjected to dideoxy sequencing (fig. 10) under conditions with which a person skilled in the art is familiar.

For the experimental examination of the replication and transcription, what is called a reporter gene was inserted in the multiple cloning site. The chloramphenicol-resistant human mitochondrial 16 S ribosomal RNA was chosen as the reporter gene. It distinguishes itself from the naturally occurring ribosomal RNA only by a modified nucleotide (polymorphism). By means of the polymerase chain reaction, a fragment having two modified oligonucleotides (primer 3, hybridized with the nucleotides 1562-1581 of the mitochondrial DNA, extended at the 5' end by the sequence CCTCTaagctt for the incorporation of a *Hind* III site; primer 4, hybridized with the nucleotides 3359-3340, extended at the 5' end by the sequence GCATTactagt for the incorporation of a *Bcl* I site) was amplified from a DNA extract of chloramphenicol-resistant HeLa cells under conditions with which a person skilled in the art is familiar. In order to ensure a correct processing of the subsequent transcript, the amplification product included the two flanking tRNA genes ($tRNA^{Val}$ and $tRNA^{Leu}$). The amplified DNA was treated with the restriction endonucleases *Hind* III and *Bcl* I, purified by precipitation and used with the pBluescript plasmid 1 treated with *Hind* III and *Bcl* I (see figs. 8, 9 and 10) in a stoichiometry of 1:1 in a ligation reaction under conditions with which a person skilled in the art is familiar. The cloning strategy is illustrated in fig. 11.

Several E. coli colonies (clones) could be isolated and characterized. For this purpose, the corresponding plasmid DNA was subjected to a dideoxy sequencing under conditions with which a person skilled in the art is familiar (see fig. 12). In order to prepare the cloned DNA for the application to cell cultures and mitochondria, the cloning insert (mitochondrial transformation plasmid) was separated by the use of the restriction endonuclease *Bsa* I from the pBluescript vector under conditions with which a person skilled in the art is familiar. Alternatively, the insert DNA could be amplified via two oligonucleotides (primers 2 and 5; nucleotide sequence of primer 5: GATCCGGTCTCATTTTATGCG) by the polymerase chain reaction. The use of S-dNTPs permitted the production of 'thionated' DNA which is stabilized over cellular nucleases. In both cases, the subsequent use of the restriction endonuclease *Bsa* I resulted in two different 5' overhangs. They are complementary to the 'hairpin loops' used in order to achieve a cyclization of the linear nucleic acid (see figs. 13a and b). The oligonucleotides are produced via chemical synthesis. As a result, they do not have phosphorylated 5' ends and have to be phosphorylated by a kinase reaction under conditions with which a person skilled in the art is familiar (in order to be able to subsequently examine the cellular transformation, [γ-^{32}P]-ATP was partially used in this reaction as substrate to radioactively label the plasmid). A majority of the 'hairpin loop' structure of the oligonucleotides forms spontaneously, since the palindromic sequence can hybridize with itself. However, dimers of the 'hairpin loops' can also be converted into monomers by denaturing them in the greatest possible volume (<0.1 μM) at 93°C for at least 5 min. and fixing them immediately in a solid matrix by freezing. Then, the oligonucleotides are slowly thawed at 4°C and then 99 % thereof are available in the desired monomeric 'hairpin loop' structure (see fig. 14).

The plasmid DNA was cyclized together with the two monomerized 'hairpin loops' (HP 1 and 2) in a reaction

batch. In this case, the molar ratio of plasmid DNA to the two 'hairpin loops' was 1:100:100 (plasmid:HP1:HP2). By using the T4 DNA ligase, the individual reactants could be combined under conditions with which a person skilled in the art is familiar (see fig. 15). The ligation products were purified by a treatment with exonuclease III (reaction conditions: 37°C, 60 min.). While nucleic acids having free 3' ends are decomposed by the nuclease, the plasmid DNA linked with the two 'hairpin loops' remains stable over the 3'-5' exonuclease activity of the enzyme. The only reaction product (see fig. 15a) was separated via a preparative agarose gel electrophoresis and purified by an electroelution or by using QIAquick (Qiagen) in accordance with the manufacturer's recommendation.

The ligation product was examined via an RFLP analysis (restriction fragment length polymorphism). For this purpose, the ligated and purified plasmid DNA was treated with the restriction endonuclease *Mae* III under conditions with which a person skilled in the art is familiar. The DNA had five cleavage sites, so that fragments of differing sizes form which can be analyzed via an agarose gel (4 %). Fig. 15b shows by way of example the *Mae* III cleavage pattern that is obtained after the ligation of the plasmid DNA with the two 'hairpin loops'. In this case, the DNA bands marked by the arrow tips represent the left and right end of the amplified (lane 1) and the linear-cyclic (lanes 2 and 3) mitochondrial plasmids.

For the conjugation of the circularized plasmid with the synthetic signal peptide of the rat ornithine transcarbamylase (H_2N-MLSNLRILLNKAALRKAHTSMVRNFRYGKPVQSQVQ-LKPRDLC-COOH), the nucleic acid was incubated with 20 times a molar excess of m-maleimidobenzoyl-N-hydroxysuccinimide ester (linkage agent) at 20°C for 60 min. (incubation medium: 50 mM potassium phosphate pH 7.8). The excess linkage agent was separated by a 'nick spin column' (Pharmacia-LKB) under conditions with which a person skilled in the art is familiar. The 'activated' nucleic

acid was conjugated by reacting the nucleic acid with 50 times the molar excess of the signal peptide at 20°C (incubation medium: 50 mM potassium phosphate pH 6.8). The reaction was stopped by the addition of 1 mM dithiothreitol after 45 min. and the conjugate was available for the experiments to come.

In order to be able to show the *in vivo* usability of the peptide-nucleic acid plasmid, the plasmid had to be incorporated into eukaryotic cells. For this purpose, a chloramphenicol-sensitive B lymphocyte or fibroblast cell culture was transfected via a lipotransfection with the peptide-nucleic acid plasmid: 1 µg of the radioactively labeled peptide-nucleic acid plasmid (the labeling was introduced as ^{32}P labeling during the kinase reaction of the 'hairpin loop' (HP1)) was pre-incubated together with 2-6 µl LipofectAmine^R (Gibco-BRL) in 200 µl serum-free Optimem^R (Gibco-BRL) (20°C, 15 min.). During the incubation the polycationic lipid of the LipfectAmine^R reagent DOSPA (2,3-dioleyloxy-N-[2-(sperminecarboxamido)-ethyl]-N,N-dimethyl-1-propaneaminiumtrifluoroacetate) forms unilamellar liposomes with the aid of the neutral lipid DOPE (dioleoylphosphatidylethanolamine), which can complex the DNA. Then, the reaction batch was added to the prepared cells, adjusted to a density of about $2.5*10^6$ cells per 0.8 ml (35 mm culture dishes, 4 h, 37°C, CO_2 incubator). The transfection medium was then replaced by 5 ml of DMEM medium (Gibco-BRL) previously supplemented by 10 % fetal calf serum and 100 µg/ml chloramphenicol. The transformation efficiency was determined by the measurement of the ^{32}P radiation of the construct. As a rule, a cellular incorporation rate of 80-85 % was measured. This means that 80-85 % of the chimerical construct were associated with the transformed cells and 15-20 % of the chimerical peptide-DNA plasmid remained in the supernatant of the transfection reaction.

After about 21-28 days, chloramphenicol-resistant colonies formed in the transformed cells. Under conditions with

which a person skilled in the art is familiar, the resistant cells were isolated and multiplied. Under conditions with which a person skilled in the art is familiar, sufficient DNA could be obtained from about $1*10^5$ cells to classify the genotypes. For this purpose, the isolated DNA was separated via agarose gel electrophoresis and transmitted to a nylon membrane (Southern blot). The nucleic acids were detected by hybridization using a specific, radioactively labeled probe (see fig. 16). In addition to the introduced circularized 'linear' vector (lanes 2 and 6) a 'in vitro' transcription (lane 3), an 'in vitro' replication (lane 4), as well as the intermediates obtained 'in vivo' (isolated nucleic acids of a transformed clone) are shown in this illustration. While the three smaller bands can be produced in vitro by incubating the circularized vector with the four nucleoside triphosphates (RNA) and a mitochondrial enzyme extract (lane 3), the formation of a dimer, circular plasmid (greatest band in lane 4) is observed in the further addition of the deoxynucleoside triphosphates to the reaction batch: an identical image yields the analysis of the nucleic acids which can be obtained from transformed cell colonies (lane 5). The fact that the greatest DNA band in lanes 4 and 5 is actually the dimeric and thus replicated mitochondrial plasmid, could be confirmed by sequence analysis.

A lipotransfection batch where the non-conjugated plasmid not linked with the signal peptide was used, served as control experiment. As expected, this plasmid was not incorporated into the mitochondria of the transfected cells and thus did not result in the formation of chloramphenicol-resistant cells. These cells stopped growth after 10 days and decayed within the following 8 to 10 days completely.

Claims

1) A chimerical peptide-nucleic acid fragment comprising:
   (a) a cell-specific, compartment-specific or membrane-specific signal peptide, with the exception of a KDEL signal sequence,
   (b) a linkage agent,
   (c) a nucleic acid (oligonucleotide),
   the signal peptide being linked via the linkage agent which via amino acids at the carboxy-terminal end of the signal peptide is linked therewith so as to ensure the appropriate nucleic acid introduction into cell organelles and cells.

2) The chimerical peptide-nucleic acid fragment according to claim 1, characterized in that the nucleic acid consists of at least two bases.

3) The chimerical peptide-nucleic acid fragment according to claim 1 or 2, characterized in that the nucleic acid has a hybridizable secondary structure.

4) The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 3, characterized in that the nucleic acid has a palindromic sequence.

5) The chimerical peptide-nucleic acid fragment according to claim 4, characterized in that the nucleic acid may form a 'hairpin loop'.

6) The chimerical peptide-nucleic acid fragment according to claim 5, characterized in that the nucleic acid may hybridize with itself and may form an overhanging 3' end or 5' end ('sticky end').

7) The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 6, characterized in that the nucleic acid is a ribonucleic acid, preferably a deoxyribonucleic acid.

8) The chimerical peptide-nucleic acid fragment according to claim 7, characterized in that the nucleic acid has chemically modified 'phosphorus thioate' linkages.

9) The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 8, characterized in that the nucleic acid carries a reactive linkage group.

10) The chimerical peptide-nucleic acid fragment according to claim 9, characterized in that the reactive linkage group contains an amino function when the linkage agent contains an amino-reactive grouping.

11) The chimerical peptide-nucleic acid fragment according to claim 9, characterized in that the reactive linkage group contains a thiol function when the linkage agent contains a thiol-reactive grouping.

12) The chimerical peptide-nucleic acid fragment according to claim 10 or 11, characterized in that the linkage grouping present is bound to the nucleic acid via at least one C2 spacer, but preferably one C6 spacer.

13) The chimerical peptide-nucleic acid fragment according to claim 12, characterized in that the linkage grouping is localized at the 3' hydroxy/phosphate terminus or at the 5' hydroxy/phosphate terminus of the nucleic acid, but preferably at the base.

14) The chimerical peptide-nucleic acid fragment according to any one of claims 10 to 13, characterized in that defined nucleic acids, antisense oligonucleotides, messenger RNAs or transcribable and/or replicatable genes are linked with the 5' end and/or 3' end.

15) The chimerical peptide-nucleic acid fragment according to claim 14, characterized in that the nucleic acid to

be linked contains chemically modified 'phosphorus thioate' linkages.

16) The chimerical peptide-nucleic acid fragment according to claim 14 or 15, characterized in that the gene to be linked contains a promoter, preferably a mitochondrial promoter.

17) The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 16, characterized in that the signal peptide has a reactive amino acid at the carboxy-terminal end, preferably a lysine or cysteine, when the linkage agent contains an amino-reactive or thiol-reactive grouping.

18) The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 17, characterized in that the signal peptide carries a cell-specific, compartment-specific or membrane-specific recognition signal.

19) The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 18, characterized in that the signal peptide has a cell-specific, compartment-specific or membrane-specific peptidase cleavage site.

20) The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 19, characterized in that the peptide consists of the compartment-specific cleavable signal peptide of the human mitochondrial ornithine transcarbamylase, extended by an artificial cysteine at the C terminus.

21) The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 20, characterized in that the linkage agent is a bifunctional, preferably heterobifunctional, cross-linker.

22) The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 21, characterized in that the linkage agent contains thiol-reactive and/or amino-reactive groupings when the signal peptide and the nucleic acid carry thiol and/or amino groups as linkage sites.

23) The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 22, characterized in that the linkage agent is m-maleimido-benzoyl-N-hydroxy-succinimide ester or a derivative thereof.

24) The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 23, characterized in that the molecule can overcome membranes with and without membrane potential by utilizing natural transport mechanisms.

25) The chimerical peptide-nucleic acid fragment in the form of a linear-cyclic plasmid, characterized in that the plasmid comprises at least one replication origin and that both ends of the nucleic acid portion are cyclized, at least one cyclic end having a modified nucleotide which via a linkage agent can be linked with a cell-specific, compartment-specific or membrane-specific signal peptide.

26) The chimerical peptide-nucleic acid fragment according to claim 25, characterized in that the nucleic acid portion further comprises at least one promoter, preferably a mitochondrial promoter, especially preferably the mitochondrial promoter of the light strand.

27) The chimerical peptide-nucleic acid fragment according to any one of claims 25 and 26, characterized in that the nucleic acid portion further comprises transcription-regulatory sequences, preferably mitochondrial transcription-regulatory sequences.

28) The chimerical peptide-nucleic acid fragment according to any one of claims 25-27, characterized in that the transcription-regulatory sequences have at least one binding site of a transcription initiation factor.

29) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 28, characterized in that the transcription-regulatory sequences have at least one binding site for the RNA synthesis apparatus, preferably the binding site for the mitochondrial transcription factor 1 and the mitochondrial RNA polymerase.

30) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 29, characterized in that the transcription-regulatory sequences are arranged in the 3' direction of the promoter.

31) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 30, characterized in that the transcription is regulated by elements of the mitochondrial H-strand and L-strand transcription control.

32) The chimerical peptide-nucleic acid fragment according to claim 31, characterized in that what is called 'conserved-sequence-blocks' of L strand transcription act as transcription control elements.

33) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 32, characterized in that the plasmid further comprises at least one transcription termination site.

34) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 33, characterized in that the transcription termination site has a binding

sequence of a mitochondrial transcription termination factor.

35) The chimerical peptide-nucleic acid fragment according to claim 34, characterized in that the transcription termination site has the binding sequence of a preferably bidirectionally acting transcription termination factor.

36) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 35, characterized in that the replication origin is a mitochondrial replication origin, preferably the replication origin of the heavy mtDNA strand having at least one 'conserved sequence block'.

37) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 36, characterized in that the plasmid further comprises at least one regulatory sequence for the replication.

38) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 37, characterized in that the regulatory sequence for the replication is a mitochondrial sequence motif.

39) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 38, characterized in that the plasmid further comprises a selection gene, preferably an antibiotic-resistance gene, preferably the oligomycin- or chloramphenicol-resistance gene.

40) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 39, characterized in that the plasmid further contains a multiple cloning site which permits the expression of 'foreign genes'.

41) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 40, characterized in that

the multiple cloning site comprises recognition sequences for restriction endonucleases which do preferably not occur in another site of the plasmid.

42) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 41, characterized in that the multiple cloning site is arranged in the 3' direction of the promoter and in the 5' direction of the transcription termination site.

43) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 42, characterized in that the multiple cloning site is arranged in the 5' direction of the selection gene.

44) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 43, characterized in that the nucleic acid fragment has (phosphorylated) ends capable of ligation.

45) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 44, characterized in that the nucleic acid fragment has 'blunt ends' or overhanging 3' ends, preferably overhanging 5' ends.

46) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 45, characterized in that the nucleic acid fragment has 4 nucleotides comprising 5' overhangs which do not have a self-homology (palindromic sequence) and are not complementary to one another either.

47) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 46, characterized in that the ends of the nucleic acid fragment are cyclized via synthetic oligonucleotides.

48) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 47, characterized in that

the overhanging 5' ends of the two oligonucleotides are complementary to one differing end of the nucleic acid each.

49) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 48, characterized in that two differing 'hairpin loops' are used for the cyclization, one being specific (complementary) to the 'left' plasmid end and the other being specific to the 'right' plasmid end of the nucleic acid.

50) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 49, characterized in that the modified nucleotide is localized preferably within the 'loop'.

51) The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 50, characterized in that the plasmid DNA is amplified enzymatically by suitable oligonucleotides which have at least one recognition sequence for a restriction endonuclease which occurs preferably in non-repeated fashion in the plasmid sequence.

52) The chimerical peptide-nucleic acid fragment according to claim 51, characterized in that the restriction endonuclease to be used generated overhanging ends, preferably 5' overhanging ends, the cleavage site being localized preferably outside the recognition sequence.

53) The chimerical peptide-nucleic acid fragment according to claim 51 or 52, characterized in that the restriction endonuclease is *Bsa* I.

54) A process for the production of a chimerical peptide-nucleic acid fragment according to any one of claims 1 to 53, characterized by the following steps:

(a) reaction of a nucleic acid (oligonucleotide) containing a functional linkage group having a linkage agent,

(b) reaction of the construct of (a) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL signal sequence, and

(c) optionally extension of the chimerical peptide-nucleic acid fragment of (b) by further DNA or RNA fragments.

55) The process according to claim 54, characterized in that the DNA in step (c) is a PCR-amplified DNA fragment containing the human mitochondrial promoter of the light strand (P_L) as well as the gene for the mitochondrial transfer RNA leucine ($tRNALeu^{(UUR)}$).

56) The process for the production of a chimerical peptide-nucleic acid fragment according to any one of claims 1 to 53, characterized by the following steps:

(a) optional extension of the nucleic acid containing a functional linkage group by further DNA or RNA fragments,

(b) reaction of the nucleic acid with functional linkage group or the extended nucleic acid of (a) with a linkage agent,

(c) reaction of the construct of (b) with amino acids at the carboxy-terminal end of a peptide containing a signal sequence, with the exception of a KDEL signal sequence.

57) The process according to claim 56, characterized in that the DNA in step (a) is a PCR-amplified DNA fragment containing the human mitochondrial promoter of the light strand (P_L) as well as the gene for the mitochondrial transfer RNA leucine ($tRNALeu^{(UUR)}$).

58) Use of the chimerical peptide-nucleic acid fragment according to any one of claims 1 to 53 for the

appropriate nucleic acid introduction into cell organelles and cells, characterized by reacting the fragment with cells or pretreated cell compartments.

59) Use according to claim 58, characterized in that the pretreated cell compartments are energized mitochondria.

60) Use of the chimerical peptide-nucleic acid fragment according to any one of claims 1 to 59 for the introduction into eukaryotic cells.

61) Use of a chimerical peptide-nucleic acid fragment according to claim 60, characterized by employing the 'particle gun' system, electroporation, microinjection or lipotransfection for the introduction into eukaryotic cells.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

CHIMERICAL PEPTIDE-NUCLEIC ACID FRAGMENT, PROCESS FOR PRODUCING THE SAME AND ITS USE FOR APPROPRIATELY INTRODUCING NUCLEIC ACIDS INTO CELL ORGANELLES AND CELLS

This is a national phase filing of the Application No. PCT/DE95/00775, which was filed with the Patent Corporation Treaty on June 11, 1995, and is entitled to priority of the German Patent Application P 44 21 079.5, filed June 16, 1994.

I. FIELD OF THE INVENTION

This invention relates to a chimerical peptide-nucleic acid fragment, the process for producing the same and its use for appropriately introducing nucleic acids into cell organelles and cells.

II. BACKGROUND OF THE INVENTION

It is now that cellular membrane systems are largely impermeable to nucleic acids. However, cell membranes can be overcome very efficiently by physical processes (transformation) and biological processes (infection). Transformation, i.e., the direction absorption of the naked nucleic acid by the cells, is preceded by cell treatment. There are various methods available for the production of these 'competent cells'. Most processes are based on the observations made by Mandel and Higa (M. Mandel *et al.*, (1970), "Calcium-dependent bacteriophage DNA infection", *J. Mol. Biol.* 53: 159-162), who could show for the first time that yields resulting from the absorption of lambda-DNA by bacteria can be increased fundamentally in the presence of calcium chloride. This method is also used successfully for the first time by Cohen *et al.* (S.N. Cohen *et al.* (1972), "Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA", *Proc. Natl. Acad. Sci. U.S.A.* 69:2110-2114) for plasmid DNA and was improved by many modifications (M. Dager *et al.* (1979), "Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells", *Gene* 6:23-28). Another transformation method is based on the observation that high-frequency alternating fields may break up cell membranes

(electroporation). This technique can be used to introduce naked DNA into not only prokaryotic cells but also eukaryotic cell systems (K. Shigekawa *et al.* (1988), "Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells", *Biotechniques* 6:742-751). Two very gentle methods of introducing DNA into eukaryotic cells were developed by Capecchi (M.R. Capecchi (1988)), "High efficiency transformation by direct microinjection of DNA into cultured mammalian cells" *Cell* 22:479-488) and Klein *et al.* (T.M. Klein *et al.* (1987), "High velocity microprojectiles for delivering nucleic acids into living cells", *Nature* 327:70-73): They are based on the direct injection of the DNA into the individual cell (microinjection), on the one hand, and on the bombardment of a cell population with microprojectiles consisting of tungsten, to the surface of which the corresponding nucleic acid was bound ('shotgun'). The biological infection methods proved their value parallel to the physical transformation of cells. They include particularly the high efficient viral introduction of nucleic acids into cells (K.L. Berkner (1988), "Development of adenovirus vectors for the expression of heterologous genes", *Biotechniques* 6:616-629; L.K. Miller (1989), "Insect baculoviruses: powerful gene expression vectors", *Bioessays* 11:91-95; B. Moss *et al.* (199), "Product review. New mammalian expression vectors", *Nature* 348:91-92) and the liposome mediated lipofection (R.J. Mannino *et al.* (1988), "Liposome mediated gene transfer", *Biotechniques* 6:682-690; P.L. Felgner *et al.* (1987), "Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure", *Proc. Natl. Acad. Sci. U.S.A.* 84:7413-7417). All methods described so far deal with the overcoming of the prokaryotic or eukaryotic plasma membrane by naked or packaged nucleic acids. While the site of action is reached already when the nucleic acid are introduced into the prokaryotic cell, further biochemical processes take place in a compartmentalized eukaryotic cell, which support the penetration of the nucleic acid into the nucleus under certain conditions (e.g. viral route of infection in the case of HIV). Analogous infective processes in which exogenous nucleic acids are actively introduced into other cell organelles (e.g., into mitochondria) have not been described so far.

In addition to the introduction of the nucleic acid into the cell and cell organelle, respectively, the transcription and above all the replication of the introduced nucleic acid

play a decisive part. In this connection, it is known that the DNA molecules may have a special property which permits duplication in a cell under certain conditions. A special structural element, the origin of the DNA replication (ori, origin), adds thereto. Its presence provides the ability of DNA replication (K.J. Marians (1992), "Prokaryotic DNA replication", *Annu. Rev. Biochem.* 61:673-719; M.L. DePamphilis (1993), "Eurkaryotic DNA replication: anatomy of an origin", *Annu. Rev. Biochem.* 62:29-63; H. Echols and M.F. Goodman (1991), "Fidelity mechanisms in DNA replication", *Annu. Rev. Biochem.* 60:477-511). The strictly controlled process of DNA replication starts in E. coli e.g., when a protein is bound (K. Geider and H. Hoffman Berling (1981), "Proteins controlling the helical structure of DNA", *Annu. Rev. Biochem.* 50:233-260) to the highly specific initiation site thus defining the starting point of a specific initiation site thus defining the starting point of a specific RNA polymerase (primase). It synthesizes a short RNA strand (~10 nucleotides, 'primer') which is complementary to one of the DNA template strands. The 3' hydroxyl group of the terminal ribonucleotide of this RNA chain serves as a 'primer" for the synthesis of new DNA by a DNA polymerase. DNA-untwisting proteins unwind the DNA double helix (J.C. Wang (1985), "DNA topoisomerases", Annu. Rev. Biochem. 54:665-697). The separated individual strands are stabilized by DNA-binding proteins as regards their conformation (J.W. Chase and K.R. Williams (1986), "Single-stranded DNA binding proteins required for DNA replication", *Annu. Rev. Biochem.* 55:103-136) to enable proper functioning of the DNA polymerases (T.S. Wang (1991), "Eukaryotic DNA polymerases", *Annu. Rev. Biochem.* 60:513-552). A multienzyme complex, the holoenzyme of DNA-polymerase-III, synthesizes the majority of the new DNA. The RNA portion of the chimerical RNA-DNA molecule is then split off the DNA polymerase III. The removal of the RNA from the newly formed DNA chains creates gaps between the DNA fragments These gaps are filled by the DNA-polymerase I which can newly synthesize DNA from a single-stranded template. While one of the two newly synthesized DNA strands is synthesized continuously (5'-3' direction, leader strand), Ogawa and Okazaki observed that a majority of the newly synthesized opposite strand (3'-5' direction, delayed strand) was synthesized from short DNA fragments (T. Ogawa and T. Okazaki (1980), "Discontinuous DNA replication", *Annu. Rev. Biochem.*

49:421-457). Here, what is called primases initiate the onset of the DNA synthesis of the opposite strand by the synthesis of several RNA primers. When the replication proceeds, these fragments are freed from their RNA primers, the gaps are closed and covalently linked with one another to give extended daughter strands by the DNA ligase. Two chromosomes form after the termination of the replication cycle.

As opposed thereto, the DNA replication is controlled by many plasmids via a replication origin which dispenses with the synthesis of the delayed strand (3'-5' direction) and can initiate the synthesis of two continuous DNA strands bidirectionally (each in the 5'-3' direction along the two templates). The precondition for a complete DNA replication is here the cyclic form of the nucleic acid. It ensure that at the end of the new synthesis of the complementary DNA strands the DNA polymerases return to the starting point again where now ligases guarantee the covalent linkage of the ends of the two newly synthesized daughter strands.

Smallpox viruses represent an interesting form of linear-cyclic nucleic acids: because of what is called 'hairpin loops' at the ends of their linear genomes they have a cyclic molecule structure while maintaining a predominantly linear conformation (D.N. Black *et al.* (1986), "Genomic relationship between capripoxviruses", *Virus Res.* 5:277-292; J.J. Esposito and J.C. Knight (1985) "Orthopoxyvirus DNA: a comparison of restriction profiles and maps", *Virology* 143:230-251). Covalently closed "hairpin" nucleic acids were not only found in smallpox viruses but also described for the ribosomal RNA from Tetrahymena (E.H. Blackburn and J.T. Gall (1978), "A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena", *J. Mol. Biol.* 120:33-53) and the genomes of the parvoviruses (S.E. Straus *et al.* (1976), "Concatemers of alternating plus and minus strands are intermediates in adenovirus-associated virus DNA synthesis", *Proc. Natl. Acad. Sci. U.S.A.* 73:742-746; P. Tattersall and D.C. Ward (1976), "Rolling hairpin model for the replication of parvovirus and linear chromosomal DNA", *Nature* 263:106-109).

However, my means of the formerly known plasmids or nucleic acid constructs it is not possible to appropriately introduce nucleic acids into cells or cell organelles via the protein import route. But this is e.g. a precondition for treating genetically changes for the mitochondrial genomes of patients suffering from neuromuscular and

neurodegenerative diseases or carrying out an appropriate mutagenesis in mitochondria or other cell organelles.

III. SUMMARY OF THE INVENTION
[TRANSLATION WILL BE PROVIDED]

IV. BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is explained particularly the figures, wherein:

Figure 1 depicts a signal peptide of the ornithine transcarbamylase of rats as well as a DNA sequence suitable for the introduction. Top: signal peptide of the ornithine transcarbamylase of rates (32 amino acids), extended by 10 N-terminal amino acids of the matured protein and an additional cysteine as linkage site. The peptide sequence is shown in the international one-letter code; middle: a partially palindromic DNA sequence suitable for the introduction and consisting of 39 nucleotides having an amino-modified T at nucleotide position 22; bottom: marked secondary structure of the oligonucleotide having an overhanging 5' end and a modified nucleotide in the vertex of the 'loop'.

Figure 2 depicts the structure of the amino-modified 2-deoxythymidine, R: nucleic acid residues.

Figure 3 depicts a diagram of chimerical peptide-nucleic acid fragment, consisting of amino-modified oligonucleotide (39 nucleotides) with marked 'hairpin loop', cross-linker and signal peptide. CL: Cross=linker.

Figure 4 the electrophoretic separation of the linkage product resulting from amino-modified oligonucleotide (39 nucleotides), m-maleimidobenzoyl-N-hydroxy-succinimide ester (MBS) and signal peptide of the ornithine transcarbamylase of rates (42 amino acids, extended by a cysteine at the C terminus).

Figure 5A depicts a flow diagram of the peptide-DNA fusion, cloning, amplification and linkage of the transcribable and processable mitochondrial tRNA gene to be introduced (S. Anderson et al. (1981), "Sequence and organization of the human mitochondrial genome", *Nature* 290:457-465). CL: cross-linker (MBS); MCS: multiple cloning site of pBluescript^{R} (Stratagene), mtTF: binding site of the

mitochondrial transcription factor; RNA-Pol: binding site of the mitochondrial RNA polymerase; tRNA Leucin: gene of the mitochondrial transfer RNA for leucine (UUR); *Sac II, Apa I, Eco RI*: sites for restriction endonucleases; the cloned mitochondrial sequences were numbered in accordance with the publishes sequence of the human mitochondrial genome (S. Anderson *et al.* (1981), "Sequence and organization of the human mitochondrial genome", *Nature* 290:457-465)

Figure 5B depicts the sequence of the cloned $tRNA^{Leu(UUR)}$ gene.

Figure 6A and 6B depict a presentation of the ^{32}p radiation of the DNA as well as the enzyme activities for adenylate kinase, cytochrome c oxidase and malate dehydrogenase (y axes) in 11 fractions (x axes) of a mitochondria-sucrose gradient density centrifugation. The portion of the particular radiation/enzyme activity, expressed as percentage of the total radiation/enzyme activity which was plotted against the gradient is illustrated. ADK: adenylate kinase; COX: cytochrome c oxidase; MDH: malate dehydrogenase.

Figure 7A and 7B depicts a presentation of the ^{32}p radiation of the DNA as well as the enzyme activities for adenylate kinase, cytochrome c oxidase and malate dehydrogenase (y axes) in 11 fractions (x axes) of a mitoplast-sucrose gradient density centrifugation. The portion of the particular radiation/enzyme activity, expressed as percentage of the total radiation/enzyme activity which was plotted against the gradient, is illustrated. ADK: adenylate kinase; COX: cytochrome c oxidase; MDH: malate dehydrogenase.

Figure 8 depicts the cloning of the nucleic acid portion of the peptide-nucleic acid plasmid into pBluescript (plasmid 1). Using the two oligonucleotides (primers 1 and 2), the gene section of nucleotide 15903 to nucleotide 677 was amplified enzymatically from mitochondrial HeLa DNA (comprises: promoter characterized by the binding sites for the mitochondrial transcription factors and the RNA polymerase; replication origin characterized by what is called 'conserved sequence blocks'; regulation of the DNA replication characterized by the 'TAS' motifs). Since the oligonucleotides contain recognition sequences for the restriction endonucleases *Xho* I and *Pst* I, the ends of the amplified nucleic acid can be modified such that they are compatible with a vector arm of pBluescript, on the one hand, and compatible with the

hybrid of the oligonucleotides MCS/TTS 1 and 2, on the other hand. In addition to a multiple cloning site, they also comprise a transcription termination sequence which is responsible for the regulated transcription. The ligation product is then transformed into *E. coli* XL 1. Following the plasmid isolation of insert-carrying *E. coli* colonies, the nucleic acids were subjected to RFLP and sequence analysis.

Figure 9 depicts the sequence of the oligonucleotides MCS/TTS 1 and 2. The oligonucleotides MCS 1 and 2 were prepared synthetically and comprise recognition sequences for nine different restriction endonucleases as well as a sequence motif which can suppress the transcription bidirectionally. The olignucleotides are complementary and can thus form a hybrid. The overhanging ends are part of the recognition sequences for the restriction endonucleases *Pst*I and *Bam* HI.

Figure 10 depicts the nucleotide sequence of the nucleic acid portion of the peptide-nucleic acid plasmid (plasmid 1).

Figure 11 depicts the cloning of the reporter gene into the nucleic acid portion of the peptide-nucleic acid plasmid into pBluescript (plasmid 2). Using the two oligonucleotides (primers 3 and 4), the gene section of nucleotide 1562 to nucleotide 3359 was amplified enzymatically from a DNA extract of a human CAP=resistant cell line (comprises: part of the 12 S rRNA gene, $tRNV^{Val}$ gene, 16 S $rRNA^{CAP+}$ gene, $tRNA^{Leu}$ gene, part of ND 1 gene). Since the oligonucleotides contain recognition sequences for the restriction endonucleases *Hind* III and *Bcl* I, the ends of the amplified nucleic acid can be modified such that they are compatible with the multiple cloning site (MCS) of the peptide-nucleic acid plasmid (plasmid 1). The ligation product is then transformed in *E. coli* XL 1 Blue. Following the plasmid isolation of insert-carrying *E. coli* colonies, the nucleic acids were subjected to the RFLP and sequence analysis and are available for the described experiments.

Figure 12 depicts the nucleotide sequence of the nucleic acid portion of the peptide-nucleic acid plasmid including the reporter gene (plasmid 2).

Figure 13A depicts the reaction run of the cyclization of the nucleic acid portion as well as the conjugation of the nucleic acid portion with a signal peptide. The nucleic acid portion of the peptide-nucleic acid plasmid can be obtained via a plasmid preparation or an enxymatic amplification. In both cases, the treatment with the

restriction endonuclease *Bsa* I results in an intermediate product capable of ligation. It can be reacted directly with the monomerized 'hairpin loops'. The reaction product is freed by an exonuclease III treatment from non-specific (non-cyclic) reaction products and educts, is purified and conjugated with the signal peptide via a cross-linker. As an alternative, one of the two 'hairpin loops' can first be conjugated with the signal peptide via a cross-linker before the cyclizing ligation reaction is carried out. A purification of the reaction product follows an exonuclease III treatment here as well.

Figure 13B depicts the structure and sequence of the 'hairpin loop' oligonucleotides HP 1 and 2.

Figure 14 depicts the monomerization of a 'hairpin loop' oligonucleotide. The synthetic 'hairpin loops' HP 1 and 2 can be monomerized by a thermal or alkaline denaturation. This figure shows a standard agarose gel: lane 1, molecular weight standard (ΦX 174 RF DNA treated with the restriction endonuclease *Hae* III), lane 2: HP 1, synthesis product; lane 3: HP 1, thermally monomerized.

Figure 15 depicts a ligation reaction between the nucleic acid portion of the peptide-nucleic acid plasmid (plasmid 2) and the 'hairpin loops' HP 1 and 2. This figure shows a standard agarose gel: lane 1, cloned nucleic acid portion of the peptide-nucleic acid portion in pBluescript treated with the restriction endonuclease *Bsa* I, lane 2: ligation of the reaction products resulting from lane 1 with the 'hairpin loops' HP 1 and 2; lane 3, treatment of the reaction production resulting from lane 2 with exonuclease III; lane 4, molecular weight standard (λDNA treated with the restriction endonucleases *HIND* III and *Eco* RI).

Figure 15B depicts the examination of the purified ligation product by a *Mae* III-RFLP analysis. This figure illustrates a standard agarose gel: lane 1, enzymatically amplified nucleic acid portion following a *Mae III* treatment; lane 2: purified ligation product of the enzymatically amplified nucleic acid portion following a *Mae* III treatment; lane 3: purified product of the plasmid DNA ligation following a *Mae* III treatment; lane 4, molecular weight standard (ΦX 174 RF DNA treated with the restriction endonuclease *Hae* III).

Figure 16 depicts the transcription and replication of the peptide-nucleic acid plasmid. This figure illustrates a standard agarose gel: lane 1, molecular weight

standard (λDNA treated with the restriction endonucleases *Hind* III and *Eco* RI); lane 2, untreated peptide-nucleic acid plasmid; lane 3: *in vitro*-obtained transcription products of the peptide-nucleic acid plasmid; lane 4: *in vitro*-obtained replication and transcription products of the peptide-nucleic acid plasmid; lane 5, *in vivo*-obtained replication and transcription products of the peptide nucleic acid plasmid; lane 6, untreated peptide-nucleic acid plasmid.

V. DETAILED DESCRIPTION OF THE INVENTION

It is the object of the present invention to develop a construct on a nucleic acid basis which permits the appropriate introduction of nucleic acids into cell and compartments of eukaryotic cells. Furthermore, a process is to be provided of how this construct can reach cell compartments or cells. In addition, the introduced nucleic acid should be such that it can also be incorporated as replicative nucleic acid via cellular protein import routes. Besides properties should be presented which result in a controlled transcription and/or replication in cells and in defined aimed compartments of cell, respectively. The process is to be used for the therapy of genetic diseases (changes of the mitochondrial genome) and for the appropriate mutagenesis in eukaryotic and prokaryotic cells. The invention is to meet the following demands:

- universal applicability
- cell-specific, compartment-specific and membrane-specific introduction behavior
- high degree of effectiveness
- low immunogenicity
- minimization of the infection risk
- the introduced nucleic acid (plasmid molecule) is to be replicatable
- the introduced nucleic acid (plasmid molecule) is to be transcribable
- the introduced nucleic acid (plasmid molecule) shall be resistant to nucleases
- the structure of the introduced nucleic acid (plasmid molecule) should be universally usable.

This problem is solved by the features of claims 1), 25), 54), 56), 58), 60) and 61). Advantageous embodiments follow from the subclaims.

In order to be able to appropriately carry a protein within a cell from the site of formation to another compartment or another cell organelle (e.g. the site of action), the protein is usually synthesized as a preprotein (R. Zimmermann et al. (1983), "Biosynthesis and assembly of nuclear-coded mitochondrial membrane proteins in Neurospora crassa", *Methods Enzymol.* 97:275-286). In addition to the matured amino acid sequence, the preprotein has what is called a signal sequence. This signal sequence is specific to the aimed compartment and enables that the preprotein can be recognized by surface receptors. The natural obstacle 'membrane" is then overcome by translocating the preprotein through the membrane by an active (several 'transport proteins' are involved in this process) or passive process (direct passage without involvement of further proteins). Thereafter, the signal sequence is usually separated on the site of action by a specific peptidase unless it is a constituent of the matured protein. The matured protein can now unfold its enzymatic activity.

The inventors have recognized that his mechanism can be utilized to appropriately transport nucleic acids across membranes. In this case, the nucleic acid is not subject to a restriction, i.e., it is possible to use every nucleic acid desired and known, respectively. For this purpose, a cell-specific, compartment-specific or membrane-specific signal sequence is linked with the desired nucleic acid, resulting in a chimerical peptide-nucleic acid fragment. In this context, it is known that the linkage between a nucleic acid and a peptide may occur via the α-amino group of a synthetic KDEL peptide, modified by ϵ-maleimidocapronic acid-N-hydroxysuccinimide ester (K. Arar *et al.* (1993), "Synthesis of oligonucleotide-peptide conjugates containing a KDEL signal sequence", *Tetrahedron Lett.* 34:8087-8090). However, this linkage strategy is completely unusable for the nucleic acid introduction into cell organelles and cells, since here the translocation should occur in analogy to the natural protein transport. Such a transport cannot be expected by clocking the α-amino group of a synthetic peptide by means of a nucleic acid. Therefore, the inventors chose linkage via a carboxy-terminal amino acid. On the one hand, this ensures a 'linear' linkage, on the other hand, the

free amino-terminal end of the signal peptide is thus available for the essential steps of the import reaction.

In order to be able to utilize the described transport mechanism also for the introduction of replicative and transcription-active nucleic acids, the nucleic acid is preferably integrated via a homologous recombination into an existing genome or is itself the carrier of the genetic elements, which ensures an autonomous initiation of replication and transcription. Only the latter variant complies with the criterion of universal applicability, since a recombination into an existing cellular genome is successful only under certain conditions and in select cells.

In this case, the use of cyclic DNA represents one possibility, since the DNA polymerases at the end of the new synthesis of the daughter strands return to the initial point thus guaranteeing a complete DNA replication. Although the use of a double-stranded cyclic plasmic meetings all physical criteria for a successful replication in every aimed compartment of the cell, this physical DNA form is confronted with the import pore size which is decisively involved in the appropriate translocation: Even the compact diameter of a superhelical plasmic can be compared with that of globular proteins, therefore, a translocation through a membrane system via the protein import route appears impossible. Here, an approach to a solution consists in the use of linear-cyclic DNA molecules having modified (cyclic) ends but only the diameter of linear DNA molecules. On the one hand, they are no obstacle for the import pore size; on the other hand, these linear-cyclic DNA molecules include all physical preconditions to be able to form replicative and transcription-active plasmids in the mitochondria.

The following is preferably required for the construction of the chimerical peptide-nucleic acid fragment according to the invention as well as for the construction of a replicative and transcription-active nucleic acid portion (plasmid):

- signal peptide and signal sequence, respectively, (cell-specific, compartment-specific, or membrane-specific)
- linkage agent
- nucleic acid (oligonucleotide) which may preferably comprise the following further information:

- information in the initiation and regulation of transcription and replication,
- information as to the termination of transcription and replication,
- multiple cloning site for a nucleic acid to be introduced (to be expressed) additionally,
- possible modifications, so that 'hairpin loops' can be added (cyclization of the ends) which permit linkage with the signal peptide.

The selection of the signal sequence depends on the membrane and membrane system, respectively, which is to be overcome and the aimed compartment of the cell (cell nucleus, mitochondrion, chloroplast) or the cell organelle which is to be obtained. Proteins which are to be introduced e.g. into one of the four mitochondrial compartments (outer mitochondrial membrane, intermembranous space, inner mitochondrial membrane, matrix space), have compartment-specific signal sequences. In general, signal sequences are chosen for the introduction of nucleic acids which contain a cell specific, compartment-specific or membrane-specific recognition signal thus directing the attached nucleic acid of its site of action (e.g., inner side of the inner mitochondrial membrane or matrix space). A selection can be made among signal sequences which can transport proteins in the presence or absence of a membrane potential. For the nucleic acid introduction, signal sequences which function irrespective of the membrane potential are preferred, e.g., the signal sequence of ornithine transcarbamylase (OTC) for the transport into the matrix space of the mitochondria (A.L. Horwich *et al.* (1983), "Molecular cloning of the cDNA coding for rate ornithine transcarbamylase", *Proc. Natl. Acad. Sci. U.S.A.* 80:4258-4262; J. P. Kraus *et al.* (1985), "A cDNA clone for the precursor of rate mitochondrial ornithine transcarbamylase: comparison of rate and human leader sequences and conservation of catalytic sites", *Nucleic. Acids. Res.* 13:943-952). Basically, the pure signal sequence suffices for the transport into the aimed compartment. However, preferable is to select signal sequences which additionally have a cell-specific or compartment-specific peptidase cleavage site. In the most favorable case, the "cleavage site' is within the

signal sequence but can also be attached thereto by additional amino acids to ensure the cleavage of the signal sequence when the aimed compartment has been reached (e.g., the signal sequence of human OTC can be prolonged by ten additional amino acids of the matured OTC). This ensures that the nucleic acid can be separated from the signal peptide in the aimed compartment, so that the action of the nucleic acid fully unfolds. The selected signal sequence is prepared biologically (purification of natural signal sequences or cloning and expression of the signal sequence in a eukaryotic or prokaryotic expression system) but preferably in a chemical-synthetic way.

In order to ensure a linear chemical linkage between nucleic acid and signal peptide, the signal peptide is linked via a linkage agent which is generally linked therewith via amino acids, preferably via amino acids having reactive side groups, preferably via an individual cysteine or lysine at the carboxy-terminal end of the signal peptide. A bifunctional cross-linker services as a linkage reagent, preferably a heterobifunctinal cross-linker which has a second reactive group, preferably an aminoreactive group, in addition to a thiol-reactive group at the signal peptide when a cysteine is used as the linkage site (e.g. m-maleinimidobenzoyl-N-hydroxy-succinimide ester, MBS and its derivatives).

The nucleic acid also has a linkage site which should be compatible with the selected cross-linker. When MBS is used, the oligonucleotide should have an amino function or thiol function. The linkage group of the nucleic acid can be introduced via the chemical synthesis of the ologonucleotide and is generally localized at the 5' end, at the 3' end, but preferably directly at a modified base, e.g., as 5' amino linker (TFA amino linker Amidite^R, 1,6-(n-trifluoroacetylamino)-hexyl-β-cyanoethyl-N,N-diisopropyl phosphoramidite, Pharmacia) or a 5' thiol linker (THIOL-C6 Phosphoramidit^R, MWG Biotech) at a free 5' hydroxy/phosphate group, as 3' amino linker (3' aminomodifier-C7-CPG-Synthesesäulen^R, MWG Biotech) at a free 3' hydroxy/phosphate group, but preferably as amino-modified base analog, preferably amino-modified deoxyrurindine (Amino-Modifier-dt^R, 5'-dimethoxy-trityl-5[N-(trifluoroacetylaminohexyl)-3-acrylimido]-2'-deoxyuridine, 3'-[2-cyanoethyl)-(N,N-diisopropyl)]phosphoramidite, Glen Research) within the sequence. In this case, the reactive group compatible with the cross-linker used is spaced from the 5' end or 3' end of the oligonucleotide or the modified base by

at least one C2-spacer unit, but preferably by a C6-spacer unit. The nucleic acid (oligonucleotide) including a reactive linkage group then comprises at least two nucleotides.

In order to increase the stability of the nucleic acid (oligonucleotide) over cellular and extracellular nucleases, the chemically synthesized nucleic acids can be protected by a sulfurizing reagent (Beaucage-Reagenz^R, MWG- Biotech). The phosphorus diester bonds of the nucleic acid are converted into phosphorus thioate bonds in the chemical synthesis. This oligonucleotide can then be used for the enzymatic amplification of nucleic acids, extended by further linkage reactions with other nucleic acids or used directly.

In order to directly use the chimerical peptide nucleic acid fragment, the nucleic acid (Oligonucleotide) should have a secondary structure that can be hybridized, preferably without internal homologies so as to be able to form a linear single-strand structure. This ensures that the nucleic acid (oligonucleotide) of the chimerical peptide-nucleic acid fragment can unfold a biochemical/therapeutic effect without further nucleic acid linkages.

However, for linkage with the signal sequence it is preferred to use nucleic acids (ogionucleotides) which have two further properties:

1. The sequence is preferably partially palindromic, has a blunt 5'-3' end ('blunt end'), an overhanging 3' end ('sticky end'), but has especially an overhanging, phosphorylated 5' end ('sticky end'), especially preferably an overhanging 5' end which comprises 4 nucleotides and has no self-homology (palindromic sequence). As a result, a stable, monomeric secondary structure ('hairpin loop') may form. The overhanging 5' end serves for linking defined nucleic acids, antisense oligonucleotides, but preferably transcribable and replicatable genes.
2. In the apex of the 'loop', the oligonucleotide carries a modified base which carries a grouping reactive with respect to the cross-linker, preferably an amino-modified 2'-deoxythymidine. In this case, the amino function of this modified base enables the linkage reaction between MBS and oligonucleotide.

The chimerical peptide-nucleic acid fragment is suitable for appropriately introducing nucleic acids into cells and cell organelles (e.g. nucleus, chloroplast), particularly for introducing ribonucleic acids (mRNA, 'antisense' oligonucleotides) and deoxyribonucleic acids (complete gene, 'antisense' oligonucleotides). It is especially suitable for the introduction of transcribable and processable genes into mitochondria, but even more suitable for the introduction of replicative, transcription-active and processable linear-cyclic nucleic acids (plasmids).

In a preferred embodiment, a transcribable gene is linked to the nucleic acid, containing the reactive linkage site or to the chimerical peptide-nucleic acid fragment. This is effected preferably the amplification of a gene, preferably a cloned gene consisting of a mitochondrial promoter, preferably the promoter of the light DNA strand (O_L, nt 490 -nt 369) and the gene to be expressed in a processable form, preferably a mitochondrial gene, preferably a mitochondrial transfer RNA, preferably the mitochondrial $tRNA^{Leu(UUR)}$ (nt 3204 - nt 3345) (S. Anderson *et al.* (1981), "Sequence and organization of the human mitochondrial genome", *Nature* 290:457-465). Following the enzymatic amplification of the gene, the linkage to the nucleic acid, containing the reactive linkage site, or to the chimerical peptide-nucleic acid fragment can be effected via a 'blunt end' ligation, but preferably a 'sticky end' ligation. For this purpose, the nucleic acid to be linked has at least one end capable of linkage, which consists preferably of a 5' overhand which comprises 4 nucleotides and has no self-homology (palindromic sequence). If both ends are to be linked with 'hairpin loops', a nucleic acid will preferably be selected which had differing 5' overhangs which comprise 4 nucleotides and have no self-homology. It is especially preferred to use nucleic acids whose 5' ends also have no homology with respect to one another. For the modification of the ends (cyclization) it is then preferred to use two different 'hairpin loops', one being specific (complementary) to the 'left" plasmid end and the other being specific to the 'right' plasmid end of the nucleic acid. In order to increase the stability of the nucleic acid over cellular and extracellular nucleases, the phosphorus diester bonds of the nucleic acid can be substituted with phosphorus thioate bonds and thus be protected if modified phosphorus thioate nucleotides have been used already in the enzymatic amplification.

A process comprising the following steps is suitable for the production of a chimerical peptide-nucleic acid fragment:

(a) Reaction of a nucleic acid (oligonucleotide), containing a functional linkage group, with a linkage agent.

(b) Reaction of the construct resulting from (a) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL signal sequence, and

(c) optional extension of the chimerical peptide-nucleic acid fragment resulting from (b) by further DNA or RNA fragments.

In another preferred embodiment, the chimerical peptide-nucleic acid fragment can be produced by the following steps:

(a) Optional extension of the nucleic acid, containing a functional linkage group, by further DNA or RNA fragments.

(b) Reaction of the nucleic acid with functional linkage group or the extended nucleic acid resulting from (a) with a linkage agent.

(c) Reaction of the construct resulting from (b) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL sequence.

In another embodiment which is a linear-cyclic nucleic acid in the form a plasmid, the selection of the nucleic acid depends on the genetic information which shall be expressed in which cell and in which aimed compartment of the cell. In this connection, nucleic acids which are to be transcribed have to have a suitable promoter. For example, if a gene is to be expressed in the mitochondrial matrix, a mitochondrial promotor can be chosen, preferably the promotor of the light mtDNA strand. The transcription is controlled in other cell compartments (e.g nucleus, chloroplast) by compartment-specific promoters.

The transcription is usually regulated by what is called transcription regulation sequences, preferably mitochondrial transcription regulation sequences. In general, these sequences comprise at least binding sites for factors which initiate the transcription (transcription initiation factor) as well as the binding site for the RNA synthesis apparatus. If a transcription is to be initiated in the mitochondria, binding sequences of

the mitochondrial transcription factors and of the RNA polymerase, particularly of the mitochondrial transcription factor 1 and the mitochondrial RNA polymerase, will be suitable. In other cell compartments (e.g. nucleus, chloroplast), the transcription can be controlled by compartment-specific transcription-regulation sequences.

In order to be able to regulate the transcription, the plasmid has transcription regulation sequences which are attached preferably in the 3' direction of the transcription initiation site (promoter). For example, if the transcription of a mitochondrial transformation plasmid is to be regulated, the control elements will be suitable for the H-strand and L-strand transcription of the mitochondrial genome, however preferable would be the so-called 'conserved sequence blocks' which terminate the transcription of the L-strand and simultaneously enable the transition of the DNA replication. In order to induce the exclusive transcription of the desired gene (optionally the desired genes in a polycistronic transcription), the transcription is discontinued on a suitable site behind the 3' end of the expressive gene/genes. This is achieved by the insertion of a suitable transcription-termination site, preferable arranged in the 3' direction to the promoter. For the regulated expression, the binding sequence for a bidirectionally acting transcription-termination factor is especially suitable in this case. For the transcription-termination in the mitochondria, a binding motif of a mitochondrial transcription-termination factor is preferably chosen here. At the same time, the formation of 'antisense RNA' of the head-to-head-linked dimeric plasmids is suppressed by the use of the transcription-termination factor binding sequence.

The selection of transformed cells can be controlled via the expression of a reporter gene. Expressive genes whose expression result in a macroscopic change of the phenotype are especially suitable as reporter or selection gene. A selection is made among genes which produce resistances to antibiotics, for example. In particular, the resistance genes for oligomycin (OLI) or chloramphenicol (Cap) are suitable for the use in a mitochondrial transformation system. In this connection, the mitochondrial chloramphenicol resistance gene appears to be a particularly suitable selection gene, since CAP-sensitive cell lines already change their phenotype at a portion of about 10% of the 16 S $rRNA^{CAP+}$ gene.

The replication of the nucleic acid can be realized by an initiation site for the DNA replication (replication origin). Therefore, the chimerical peptide-nucleic acid fragment in the form of a plasmid has to have at least one replication origin. In this connection, the orientation of the replication origin can be arranged irrespective of the expressive gene (genes), but preferably the replication origin is arranged in the 3' direction of the promoter. A suitable replication origin for a mitochondrial transformation plasmid would be a mitochondrial replication origin. In particular, the origin of replication of the heavy mtDNA strand is suitable in this case. It preferably has at least one 'conserved sequence block'. The replication can be controlled via what is called regulation sequences for the replication. For this purpose, the plasmid has to have at least one such sequence motif which is preferably arranged in the 3' direction of the promoter and the replication origin. Of the replication in the mitochondria is to be regulated, a mitochondrial replication regulation sequence will be especially suitable. It is preferred to use a motif which comprises at least one of the 'termination associated sequences'. In other cell compartments (e.g. nucleus, chloroplast), the replication is initiated at least via one compartment-specific replication origin and controlled via compartment-specific replication origin and controlled via compartment-specific replication regulation sequences.

In order to permit cloning of different genes into the plasmid molecule, the plasmid nucleic acid also has to have a suitable cloning module (multiple cloning site) which has the most widely differing recognition sequences for restriction endonucleases. Here, rare recognition sequences which do not occur on other sites of the plasmid are especially suitable. The cloning module can be incorporated into any site of the transformation plasmid. If the region of the cloning site is to be integrated into the transcription of the selection gene, the insertion of the multiple cloning site in the 3' direction of the promoter and in the 5' direction of the transcription termination site will be suitable. The integration of the multiple cloning site in the 5' direction of the selection gene is especially suitable, since in this case the use of the selection system is simultaneously accompanied by transcription of the region of the multiple cloning site.

In order to permit the autonomous replication in every aimed compartment of a cell when a nucleic acid is used, it has to be ensured that, after the synthesis of the

daughter strand, the DNA replication enzymes return to the synthesis starting point again to guarantee the covalent linkage of the 3' end with the 5' end of the newly synthesized daughter stand by corresponding enzymes. For this purpose, a linear nucleic acid plasmid is suitable which can be converted into a cyclic nucleic acid. The plasmid ends can be cyclized via the use of what is called ligation-capable (phosphorylated) end of nucleic acid. For this purpose, the use of a 'blunt end' nucleic acid or a nucleic having a overhanging 3' ends, but preferably a nucleic acid having overhanging 5' ends is particularly suitable. In this case, the overhanging ends should comprise at least one nucleotide. However, it is preferred to use overhanging 5' ends which are formed of four nucleotides. They have preferably no self-homology (palindromic sequence) and are also preferably not complementary to one another in order to suppress the formation of dimers in a subsequent nucleic acid linkage.

The cyclization of the prepared plasmid ends is arranged by synthetic oligonucleotides. They have a partial self-homology (partially palindromic sequence) and are thus capable to form what is called 'hairpin loop' structures. The partially palindromic sequence results in the formation of a stable, preferably monomeric secondary structure ('hairpin loop') having a blunt 5'-3' end (blunt end), and overhanging 3' end ('sticky end'), but preferably an overhanging 5' end. These oligonucleotides are especially preferred when they have a phosphorylated 5' end. When synthetic oligonucleotides having 'hairpin loop' structure are used, the linear plasmid DNA can be converted into a linear-cyclic system. The ends of the two oligonucleotides are preferably complementary to one end of the prepared plasmid nucleic acid each. For this purpose, two different 'hairpin loops' are preferably used, one being specific (complementary) to the 'right' plasmid end to suppress the dimer formation. At least one of the two 'hairpin loop' oligonucleotides may have at least one modified nucleotide. It guarantees the linkage site to a signal peptide, so that the nucleic acid transport can be arranged via the protein import route. In the model case, this linkage site (modified nucleotide) is placed at one of the unpaired positions of the 'loop'. A chemically reactive group, particularly an amino or thiol function is especially suitable as linkage site.

In order to prepare the ends of the transformation plasmid of the modification (cyclization), it has to be ensured that the plasmid ends are complementary to the ends of the oligonucleotides ('hairpin loops'). On the one hand, this succeeds by amplifying the plasmid DNA with suitable oligonucleotides which ave at least one recognition sequence for a restriction endonuclease. In this case, recognition sequences for restriction endonucleases are suitable which do not occur repeatedly in the plasmid sequence. Especially suitable is the use of recognition sequences for restriction endonucleases generating overhanging ends ('sticky ends'), particularly those which produce overhanging 5' ends, preferably outside the own recognition sequence. In this connection, the recognition sequence for the restriction endonuclease *Bsa* I ($GGTCTCN_1N_5$) is especially suitable. On the other ahdn, the use of a cloned nucleic acid which already has the recognition sequences for a restriction endonuclease, preferably *Bsa* I, is suitable. As a result, the enzymatic amplification can be omitted and the nucleic acid obtained by plasmid preparation/restriction enzyme treatment can be used directly. It is preferred that the cloned nucleic acid already includes the recognition sequence for the restriction endonuclease *Bsa* I at both ends.

Various methods are available for purifying the transformation plasmid. Here, the main objection is to separate the cyclic plasmid molecule from the unreacted enducts. The use of DNA-degrading enzymes are proved to be suitable in this connection. In particular, it is recommended to use enzymes which ave a 5'-3' or 3'-5' exonuclease activity. Particularly the use of the exonuclease III leads to the complete hydrolysis of unreacted educts while the cyclic plasmid DNA remains intact (no free 5' ends or 3' ends). The reaction products can be purified either via electrophoretic or chromatographic processes but also by precipitation. A selection can be made among different purification processes. On the one hand, the cyclic nucleic acid conjugated with the linkage agent and the signal peptide can be treated with an exonuclease, preferably exonuclease III, and then be purified via chromatographic electrophoretic purification and precipitation, respectively. On the other hand, the cyclic plasmid DNA can also be treated with an exonuclease, preferably exonuclease III, be purified and subsequently be conjugated with the linkage agent and the signal peptide and be purified via a chromatographic, electrophoretic purification and precipitation, respectively.

The linkage with a signal peptide can be realized by means of modified oligonucleotides. This peptide directs *in vivo* the transformation plasmid into the desired cell compartment. To this end, either the transformation plasmid can first be reacted with the modified oligonucleotide (ligation) and then the conjugation with the linkage agent and the signal peptide can take place or the modified oligonucleotide is first conjugated with the linkage agent and the signal peptide an then be used for the cyclizing the transformation plasmid ends (ligation).

The transformation system (cellular transformation) can overcome the cell membrane by various methods. Here, 'particle gun' system or microinjection are suitable, but electroporation and lipotransfection are preferred. All methods ensure the introduction of the linear-cyclic peptide nucleic acid plasmid into the cytosol of the cell from where the plasmid is directed to its site of action (aimed compartment) by the conjugated signal peptide.

As compared to the prior art transformation and infection methods, mentioned in the introductory part of the description, this process offers, for the first time, the possibility of appropriately introducing nucleic acids into cells and cell organelles. The selection of the signal sequence can determine the aimed compartment which is to be reached in this case (cytosol, nucleus, mitochondrion, chloroplast, etc.). Along with the compartment-specific and cell-specific introduction behavior, this process distinguishes itself by its universal applicability. Both prokaryotic and eukaryotic cells and cell systems can be treated with the translocation vector. Since a natural transport system of the membranes is used for the appropriate introduction, the treatment of cells or cell organelles with membrane-permeabilizing agents becomes superfluous (e.g. calcium chloride method, see above).

When a replicative and transcription-active nucleic acid is used, the plasmid does not unfold its full size until the first replication cycle has been completed: As a genuine cyclic plasmid (artificial chromosome) it now has the double genetic information (head-to-head linked plasmid dimers). In particular with respect to the use of this system for a somatic gene therapy, this behavior is induced intentionally and of decisive importance, since the genes to be expressed have to compete with the defect genes of the cells. In addition to this highest possible effectiveness, the system distinguishes itself through the

fact that it does not have to be integrated into a genome via a recombination step, such as retroviral systems, so as to become replicative. As a result, uncontrollable side-effects (undesired recombination) are already suppressed to the highest possible degree from the start. Therefore the application of this plasmid system can be expected without great safety risk.

The below examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

VI. EXAMPLES

A. Example 1: Introduction of a Chimerical Peptide-Nucleic Acid Fragment Into the Mitochondria

The overcoming of the mitochondrial double membrane system with a DNA translocation vector was studied to prove that nucleic acids can be transported appropriately across membranes by the above-described process. For this purpose, the mitochondrial signal sequence of the ornithine transcarbamylase (A.L. Horwich *et al.* (1983), "Molecular cloning of the cDNA coding for rat ornithine transcarbamylase", *Proc. Natl. Aca. Sci. U.S.A.* 80:4258-4262) (enzyme of urea cycle, naturally localized in the matrix of the mitochondria) was chemically prepared and purified. The original sequence was extended by a cysteine at the C terminus as reactive group for the subsequent linkage with the DNA (see fig. 1). This ensured that the heterobifunctional cross-linker (MBS) can only be linked with the thiol group of the only cysteine. A DNA oligonucleotide (39 nucleotides) were chosen as linkage partner. It distinguishes itself by two special features:

1. The sequence is partially palindromic and has and overhanging, phosphorylated 5' end (see fig. 1). As a result, what is called a 'hairpin loop' can form. The overhanging 5' end serves for ligating to this oligonucleotide defined nucleic acids which can then be imported into the mitochonndria.
2. The oligonucleotide carries a modified base in the vertex of the 'loop' (see fig. 1). In this case, an amino-modified 2'-deoxythymidine is concerned (see fig. 2). Here, the amino function of the modified bases in this connection enables the linkage reaction between MBS and oligonucleotide.

The tree reaction partners (oligonucleotide, MBS and peptide) are linked in individual reaction steps. Firstly the oligonucleotide (50 pmoles) is reacted in a buffer (100 μl; 50 mM potassium phosphate, pH 7.6) with MBS (10 nmoles dissolved in DMSO) (reaction time: 60 min.; reaction temperature: 20°). Unreacted MBS is separated via a Nick-spin column^R, (Sephadex G 50, Pharmacia) which was equilibrated with 50 mM of potassium phosphate (pH 6.0). The eluate contains the desired reaction step with the peptide (2.5 nmoles) (reaction time: 60 min.; reaction temperature 20°C). The reaction was stopped by the addition of dithiothreitol (2 mM). The linkage product (chimera, see fig. 3) was separated via a preparative gel electrophoresis of unreacted educts and isolated from the gel by electroelution (see fig. 4). Differing nucleic acids can now be linked by simple ligation to the overhanging 5' end of the oligonucleotide.

A 283 bp long double-stranded DNA (dsDNA) was amplified via an enzymatic reaction (PCR) in the below experiment. For this purpose, a DNA fragment cloned in to pBluescript^R (Stratagene) served as template DNA, which fragment in addition to the human mitochondrial promoter of the light strand (P_L, nt 902 - nt 369) included the gene for the mitochondrial transfer RNA leucine ($tRNA^{Leuc(UUR)}$, nt 3204 - nt 4126) (see fig. 5). Two oligonucleotides served as amplification primers, primer 1 having a non-complementary 5' end (see fig. 5). The dsDNA was modified by the 3'-5' exonuclease activity of the T4 DNA polymerase (incubation in the presence of 1 mM dGTP) which can produce overhanging 5' ends under conditions with which a person skilled in the art

is familiar (C. Aslanidis *et al.* (1990), "Ligation-independent cloning of PCR products (LIC-PCR)", *Nucleic. Acids Res.* 18:6069-6074).

Together with the previously conjugated peptide-MBS oligonucleotide the PCR-amplified DNA could be joined using the T4 DNA ligase. In order to be able to easily detect the linkage partners after the introduction into the mitochondria, the free 5'-OH group of the ligated DNA was phosphorylated radioactively by an enzymatic reaction (A. Novogrodsky *et al.* (1966), "The enzymatic phosphorylation of ribonucleic acid and deoxyribonucleic acid, I. Phosphorylation at 5'-hydroxyl termini", *J. Biol. Chem.* 241:2923-2932; A. Novogrodsky *et al.* (1966), "The enzymatic phosphorylation of ribonucleic acid and deoxyribonucleic acid. I. Further properties of the 5'-hydroxy polynucleotide kinase", *J. Biol. Chem.* 241:2933-2943).

A fresh rate liver was comminuted for the isolation of mitochondria, suspended in 25 mM HEPES, 250 mM saccharose, 2 mM EDTA, 52 μM BSA and homogenized in a glass homogenizer (50 ml). Cell membranes, cellular debris and nuclei were centrifuged of fat 3000 g and the supernatant was prepared for another centrifugation. For this purpose, the supernatant was placed in cooled centrifuge cups and centrifuged at 8000 g. The isolation mitochondria were resuspended in 200 ml of the same buffer and centrifuged again at 8000 g. The purified mitochondria pellet was resuspended in an equal volume of the same buffer and energized by the addition of 25 mM succinate, 25 mM pyruvate and 15 mM malate. The protein content of the suspension was determined by a Bradford Testkit^R (Pierce). 200 μg of mitochondrial protein (energized mitochondria) were incubated together with 10 pmoles of the chimera at 37°C for 60 min. (0.6 M sorbitol 10 mM potassium phosphate pH 7.4, 1 mM ATP, 2 mM $MgCL_2$, 1 % BSA). The mitochondria were reisolated by centrifugation at 8000 g, resuspended in 0.6 M sorbitol, 10 mM potassium phosphate pH 7.4, 2 mM $MgCL_2$, 1 % BSA, 10 U/ml DNAse I and incubated at 37°C for 30 min. This washing step was repeated twice to remove non-specifically adhering molecules. For proving that the chimera is associated with the mitochondria, the re-isolated mitochondria were purified via sucrose gradient density centrifugation. The individual fractions of the gradient were analyzed to localize the chimera and the mitochondria. The adenylate kinase which determines cytochrome-c oxidate and malate dehydrogenase activity was used as marker for the

mitochondria, while the chimera could be identified via the ^{32}p radiation measurement (see fig. 6). An analog experiment for determining the non-specific DNA introduction was carried out with the same DNA which was not linked with the signal peptide (see fig. 6). It was derived from the measurements that 65% of the chimera used segregated specifically with the mitochondria, whereas the non-specific DNA incorporation was less than 5% of the DNA used. In order to show that the chimera is not only associated with the surface of the mitochondria (membrane, import receptor), the re-isolated mitochondria were not fractioned into the three compartments of outer mitochondria membrane/intermembranous space, inner mitochondrial membrane and matrix space. For this purpose, the mitochondria were incubated with digitonin (final concentration: 1.2% w/v digitonin) and the resulting mitoplasts were separated via a sucrose gradient density centrifugation, collected in fractions and the activities of marker enzymes (adenylate kinase: intermembranous space, cytochrome c oxidase: inner mitochondria membrane; malate dehydrogenase; matrix space) were determined according to Schnaitman and Grennawalt (C. Schnaitman *et al.* (1968), "Enzymatic properties of the inner and outer membranes of rat liver mitochondria", *J. Cell Biol.* 38:158-175; C. Schnaitman *et al.* (1967), "The submitochondrial localization of monoamine oxidase. An enzymatic marker for the outer membrane of rat liver mitochondria", *J. Cell Biol.* 32: 719-735) (see fig. 7). An analog experiment for determining the non-specific DNA incorporation was carried out with the same DNA which was not linked with the signal peptide (see fig. 7). It was derived from the measurements that 45% of the chimera are associated with the mitoplasts, whereas the non-specifically adhering DNA could be assessed to be less than 3%. The isolated mitoplasts (less of the outer membrane and the intermembranous space) were lyzed by Lubron^{R} (0.16 mg/mg protein; ICN) and separated into the compartments of inner mitochondrial membrane (pellet) and matrix space (supernatant) by ultracentrifugation at 144,000 g. The compartments were assigned via the measurement of the activities of the cytochrome c oxidase (inner mitochondrial membrane) and the malate dehydrogenase (matrix space). The chimera was measured via the detection of the ^{32}p radiation in the scintillation counter and the result was 75% segregation with the matrix of the mitochondria, while 25% of the

chimera remained associated with the inner membrane of the mitochondria (incomplete translocation).

B. Example 2: Incorporation of a Replicative and Transcription-Active Chimerical Peptide-Nucleic Acid Fragment (Plasmid) Into the Mitochondria of Living Cells

In order to prove that a linear peptide-nucleic acid plasmid having cyclic ends ('hairpin loops') can overcome membranes *in vivo* via the protein import route and can be transcribed and replicated in spite of the chemical linkage with a signal peptide, the transcription and replication behavior were studies after the transfection of cells and the import into the matrix of the mitochondria. For this purpose, the signal peptide of the mitochondrial ornithine transcarbamylase was prepared synthetically, purified and linked with a nucleic acid plasmid.

Precondition for the examination of the correct transcription and replication behavior is the physical structure of the plasmid: for the experiment described below, a 3232 bp long double-stranded vector DNA (dsDNA) was cloned into pBluescript^{R} (Stratagene). For this purpose, the region of the mitochondrial genome was amplified via two modified oligonucleotides (primer 1, hybridized with the nucleotides 15903-15924 of the human mtDNA, includes at the 5' end an extension by the sequence TGTAGctgcag for the incorporation of a *PstI* site; primer 2, hybridized with the nucleotides 677-657 of the human mtDNA, includes at the 5' end an extension by the sequence TTGCATGctcgagGGTCTCAGGG for the incorporation of the *XhoI* site), which comprised the promoter of the light DNA strand, the regulation motifs for the transcription (CSBs, 'conserved sequence blocks') as well as the regulation site for the DNA replication ('TAS', termination associated sequences, (D.C. Wallace (1989), "Report of the committee on human mitochondrial DNA", Cytogenet. *Cell Genet.* 51:612-621) (see fig. 8). A multiple cloning site (MCS/TTS) was produced via a chemical synthesis of two complementary oligonucleotides (MCS/TTS 1 and 2) which contain the recognition sequences for various restriction endonucleases (see fig. 9). Under conditions with which a person skilled the art is familiar, the two oligonucleotides form hybrids which, after the phosphorylation with T4 DNA polynucleotide kinase, can be used for the ligation. In this connection, the hybrids distinguish themselves by 5' and 3' single-stranded-overhanging ends which are

complementary to a *Pst* I, on the one hand, and are complementary to a *Bam* HI site, on the other hand (see fig. 9). Together with the multiple cloning site, the synthetic oligonucleotides MCS/TTS 1 and 2 also comprise a bidirectional mitochondrial transcription termination sequence (see fig. 9). It is arranged in the 3' direction of the MCS and ensures that the transcription on this site is discontinued thus correctly forming terminated transcripts forming. This sequence motif also ensures that in the cyclic plasmid system no 'antisense RNA' is expressed. The ligation reaction between pBluescript, PCR-amplified fragment and the MCS/TTS hybrids took place in a stoichiometry of 1;2:2 under conditions with which a person skilled in the art is familiar. After the transformation, several E. coli colonies (clones) could be isolated and characterized. For this purpose, the corresponding plasmid DNA was subjected to dideoxy sequencing (fig. 10) under conditions with which a person skilled in the art is familiar.

For the experimental examination of the replication and transcription, what is called a reporter gene was inserted in the multiple cloning site. The chloramphenicol-resistant human mitochondrial 16 S ribosomal RNA was chosen as the reporter gene. It distinguishes itself from the naturally occurring ribosomal RNA only by a modified nucleotide (polymorphism). By means of the polymerase chain reaction, a fragment having two modified ologonucleotides (primer 3, hybridized with the nucleotides 1562-1581 of the mitochondrial DNA, extended at the 5' end of the sequence CCTCTaagctt for the incorporation of a *Hind* III site; primer 4, hybridized with the nucleotides 3359-3340, extended at the 5' end of the sequence GCATTactagt for the incorporation of a *Bcl* I site) was amplified from a DNA extract of chloramphenicol-resistant HeLa cells under conditions with which a person skilled in the art is familiar. In order to ensure a correct processing of the subsequent transcript, the amplification product included the two flanking tRNA genes ($tRNA^{Val}$ and $tRNA^{Leu}$). The amplified DNA was treated with the restriction endonucleases *Hind* II and *Bcl* I, purified by precipitation and used with the pBluescript plasmid 1 treated with *Hind* III and *Bcl* I (see figs. 8, 9 and 10) in a stoichiometry of 1:1 in a ligation reaction under conditions with which a person skilled in the art is familiar. The cloning strategy is illustrated in fig. 11.

Several E. coli colonies (clones) could be isolated and characterized. For this purpose, the corresponding plasmid DNA was subjected to a dideoxy sequencing under conditions with which a person skilled in the art is familiar (see fig. 12). In order to prepare the cloned DNA for the application to cell cultures and mitochondria, the cloning insert (mitochondrial transformation plasmid) was separated by the use of the restriction endonuclease *Bsa* I from the pBluescript vector under conditions with which a person skilled in the art is familiar. Alternatively, the insert DNA could be amplified via two oligonucleotides (primers 2 and 5; nucleotide sequence of primer 5: GATCCGGTCTCATTTTATGCG) by the polymerase chain reaction. The use of S-dNTPs permitted the production of 'thionated' DNA which is stabilized over cellular nucleases. In both cases, the subsequent use of the restriction endonuclease *Bsa* I resulted in two different 5' overhangs. They are complementary to the 'hairpin loops' used in order to achieve a cyclization of the linear nucleic acid (see figs. 13a and b). The oligonucleotides are produced via chemical synthesis. As a result, they do not have phosphorylated 5' ends and have to be phosphorylated by a kinase reaction under conditions with which a person skilled in the art is familiar (in order to be able to subsequently examine the cellular transformation, [γ-^{32}P]-ATP was partially used in this reaction as a substrate to radioactively label the plasmid). A majority of the 'hairpin loop' structure of the oligonucleotides forms spontaneously, since the palindromic sequence can hybridize with itself. However, dimers of the 'hairpin loops' can also be converted into monomers by denaturing them in the greatest possible volume (<0.1 μM) at 93°C for at least 5 min. and fixing them immediately in a solid matrix by freezing. Then, the oligonucleotides are slowly thawed at 4°C and then 99% thereof are available in the desired monomeric 'hairpin loop' structure (see fig. 14).

The plasmid DNA was cyclized together with the two monomerized 'hairpin loops' (HP 1 and 2) in a reaction batch. In this case, the molar ratio plasmid DNA to the two 'hairpin loops' was 1:100:100 (plasmid:HP1:HP2). By using the T4 DNA ligase, the individual reactants could be combined under conditions with which a person skilled the art is familiar (see fig. 15). The ligation products were purified by a treatment with exonuclease III (reaction conditions: 37°C, 50 min.). While nucleic acids having free 3' ends are decomposed by the nuclease, the plasmid DNA linked with

the two 'hairpin loops' remains stable over the 3'-5' exonuclease activity of the enzyme. The only reaction product (see fig. 15a) was separated via a preparative agarose gel electrophoresis and purified by an electroelution or by using QIAquick (Qiagen) in accordance with the manufacturer's recommendation.

The ligation product was examined via an RFLP analysis (restriction fragment length polymorphism). For this purpose, the ligated and purified plasmid DNA was treated with the restriction endonuclease *Mae* III under conditions with which a person skilled in the art is familiar. The DNA had five cleavage sites, so that fragments of differing dizes form which can be analyzed via an agarose gel (4%). Fig. 15b shows by way of example the *Mae* III cleavage pattern that is obtained after the ligation of the plasmid DNA with the two 'hairpin loops'. In this case, the DNA bands marked by the arrow tips represent the left and right end of the amplified (lane 1) and the linear-cyclic (lanes 2 and 3) mitochondrial plasmids.

For the conjugation of the circularized plasmid with teh synthetic signal peptide of the rate orninthine transcarbamylase (H_2N-MLSNLRILLNKAALRKAHTSMVRNFRYGKPVQSQVQ-LKPRDLC-COOH), the nucleic acid was incubated with 20 times a molar excess of m-maleimidobenzoyl-N-hydroxysuccinimide ester (linage agent) at 20°C for 60 min. (incubation medium: 50 mM potassium phosphate pH 7.8 The excess linkage agent was separated by a 'nick spin column' (Pharmacia-LKB) under conditions with which a person skilled in the art is familiar. The 'activated' nucleic acid was conjugated by reacting the nucleic acid with 50 times the molar excess of the signal peptide at 20°C (incubation medium: 50 mM potassium phosphate pH 6.8). The reaction was stopped by the addition of 1 mM dithiothreitol after 45 min. and the conjugate was available for the experiments to come.

In order to be able to show the *in vivo* usability of the peptide-nucleic acid plasmid, the plasmid had to be incorporated into eukaryotic cells. For this purpose, a chloramphenicol-sensitive B lymphocyte or fibroblast cell culture was transfected via a lipotransfection with the peptide-nucleic acid plasmid (the labeling was introduced at ^{32}p labeling during the kinase reaction of the 'hairpin loop' (HP1)) was pre-incubated together with 2-6 μl serum-free Optimem^R (Gibco-BRL) (20°C, 15 min.). During the incubation the polycationic lipid of the LipfectAmine^R reagent DOSPA (2,3-dioleyloxy-

N-[2-(sperminecarboxamido)-ethyl]-N,N-dimethyl-1-propaneaminiumtrifluoroacetate) forms unillamellar liposomes with the aid of the neutral lipid DOPE (dioleoylphosphatidylethanolamine), which can complex the DNA. Then, the reaction batch was added to the prepared cells, adjusted to a density of about $2.5*10^6$ cells per 0.8 ml (35 mm culture dishes, 4 h, 37°C, CO_2 incubator). The transfection medium was then replaced by 5 ml of DMEM medium (Gibco-BRL) previously supplemented by 10% fetal calf serum and 100 µg/ml chloramphenicol. The transformation efficiency was determined by the measurement of the ^{32}P radiation of the construct. As a rule, a cellular incorporation rate of 80-85% of the chimerical construct were associated with the transformed cells and 15-20% of the chimerical peptide-DNA plasmid remained in the supernatant of the transfection reaction.

After about 21-28 days, chloramphenicol-resistant colonies formed in the transformed cells. Under conditions with which a person skilled in the art is familiar, the resistant cells were isolated and multiplied. Under conditions with which a person skilled in the art is familiar, sufficient DNA could be obtained from about $1*10^5$ cells to classify the genotypes. For this purpose, the isolated DNA was separated via agarose gel electrophoresis and transmitted to a nylon membrane (Southern blot). The nucleic acids were detected by hybridization using a specific, radioactively labeled probe (see fig. 16). In addition to the introduced circularized 'linear' vector (lanes 2 and 6) an 'in vitro' transcription (lane 3), an 'in vitro' replication (lane 4), as well as the intermediates obtained 'in vivo' (isolated nucleic acids of a transformed clone) are shown in this illustration. While the three smaller bands can be produced in vitro by incubating the circularized vector with the four nucleoside triphosphates (RNA) and a mitochondrial enzyme extract (lane 3), the formation of a dimer, circular plasmid (greatest band in lane 4) is observed in the further addition of the deoxynucleoside triphosphates to the reaction batch; an identical image yields the analysis of the nucleic acids which can be obtained from transformed cell colonies (lane 5). The fact that the greatest DNA band in lanes 4 and 5 is actually the dimeric and thus replicated mitochondrial plasmid, could be confirmed by sequence analysis.

A lipotransfection batch were the non-conjugated plasmid not linked with the signal peptide was sued, served as a control experiment. As expected, this plasmid was

not incorporated into the mitochondria of the transfected cells and thus did not result in teh formation of chloramphenicol-resistant cells. These cells stopped growth after 10 days and decayed within the following 8 to 10 days completely.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.

CLAIMS

WHAT IS CLAIMED:

1. A chimerical peptide-nucleic acid fragment comprising:

(a) a cell-specific, compartment-specific or membrane-specific signal peptide, with the exception of a KDEL signal sequence,

(b) a linkage agent,

(c) a nucleic acid (oligonucleotide),

the signal peptide being linked via the linkage agent which via amino acids at the carboxy-terminal end of the signal peptide is linked therewith so as to ensure the appropriate nucleic acid introduction into cell organelles and cells.

2. The chimerical peptide-nucleic acid fragment according to claim 1, characterized in that the nucleic acid consists of at least two bases.

3. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 2, characterized in that the nucleic acid has a secondary structure.

4. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 3, characterized in that the nucleic acid has a palindromic sequence.

5. The chimerical peptide-nucleic acid fragment according to claim 4, characterized in that the nucleic acid may form a "hairpin loop".

6. The chimerical peptide-nucleic acid fragment according to claim 5, characterized in that the nucleic acid may hybridize with itself and may form an overhanging 3' end or 5' end ('sticky end').

7. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 6, characterized in that the nucleic acid is a ribonucleic acid, preferably a deoxyribonucleic acid.

8. The chimerical peptide-nucleic acid fragment according to claim 7, characterized in that the nucleic acid has chemically modified 'phosphorus thioate' linkages.

9. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 8, characterized in that the nucleic acid carries a reactive linkage group.

10. The chimerical peptide-nucleic acid fragment according to claim 9, characterized in that the reactive linkage group contains an amino function when the linkage agent contains an amino-reactive grouping.

11. The chimerical peptide-nucleic acid fragment according to claim 9, characterized in that the reactive linkage group contains a thiol function when the linkage agent contains a thiol-reactive grouping.

12. The chimerical peptide-nucleic acid fragment according to claim 10 or 11, characterized in that the linkage grouping present is bound to the nucleic acid via at least one C2 spacer, but preferably one C6 spacer.

13. The chimerical peptide-nucleic acid fragment according to claim 12, characterized in that the linkage grouping is localized at the 3' hydroxy/phosphate terminus or at the 5' hydroxy/phosphate terminus of the nucleic acid, but preferably at the base.

14. The chimerical peptide-nucleic acid fragment according to any one of claims 10 to 13, characterized in that defined nucleic acids, antisense oligonucleotides, messenger RNAs or transcribable and/or replicatable genes are linked with the 5' end and/or 3' end.

15. The chimerical peptide-nucleic acid fragment according to claim 14, characterized in that the nucleic acid to be linked contains chemically modified 'phosphorus thioate' linkages.

16. The chimerical peptide-nucleic acid fragment according to claim 14 to 15, characterized in that the gene be linked contains a promotor, preferably a mitochondrial promoter.

17. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 16, characterized in that the signal peptide has a reactive amino acid at the carboxy-terminal end, preferably a lysine or cysteine, when the linkage agent contains an amino-reactive or thiol-reactive grouping.

18. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 17, characterized in that the signal peptide carries a cell-specific, compartment-specific or membrane-specific recognition signal.

19. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 18, characterized in that the signal peptide has a cell-specific, compartment-specific or membrane-specific peptidase cleavage site.

20. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 19, characterized in that the peptide consists of the compartment-specific cleavable signal peptide of the human mitochondrial ornithine transcarbamylase, extended by an artificial cysteine at the C terminus.

21. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 20, characterized in that the linkage agent is a bifunctional, preferably heterobifunctional cross-linker.

22. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 21, characterized in that the linkage agent contains thiol-reactive and/or amino-reactive groupings when the signal peptide and the nucleic acid carry thiol and/or amino groups as linkage sites.

23. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 22, characterized in that the linkage agent is m-maleimido-benzoyl-N-hydroxy-succinimide ester or a derivative thereof.

24. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 23, characterized in that the molecule can overcome membranes with and without membrane potential by utilizing natural transport mechanisms.

25. The chimerical peptide-nucleic acid fragment in the form of a linear-cyclic plasmid, characterized in that the plasmid comprises at least one replication origin and that both ends of the nucleic acid portion are cyclized, at least one cyclic end having a modified nucleotide which via a linkage agent can be liked with a cell-specific, compartment-specific or membrane-specific signal peptide.

26. The chimerical peptide-nucleic acid fragment according to claim 25, characterized in that the nucleic acid portion further comprises at least one promoter, preferably a mitochondrial promoter, especially preferably the mitochondrial promoter of the light strand.

27. The chimerical peptide-nucleic acid fragment according to any one of claims 25 and 26, characterized in that the nucleic acid portion further comprises transcription-regulatory sequences, preferably mitochondrial transcription-regulatory sequences.

28. The chimerical peptide-nucleic acid fragment according to any one of Claims 25-27, characterized in that the transcription-regulatory sequences have at least one binding site of a transcription initiation factor.

29. The chimerical peptide-nucleic acid fragment according to any one of Claims 25 to 28, characterized in that the transcription-regulatory sequences have at least one binding site for the RNA synthesis apparatus, preferably the binding site for the mitochondrial transcription factor 1 and the mitochondrial RNA polymerase.

30. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 29, characterized in that the transcription-regulatory sequences are arranged in the 3' direction of the promoter.

31. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 30, characterized in that the transcription is regulated by elements of the mitochondrial H-strand and L-strand transcription control.

32. The chimerical peptide-nucleic acid fragment according to claim 31, characterized in that what is called 'conserved-sequence-blocks' of L-strand transcription act as transcription control elements.

33. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 32, characterized in that the plasmid further comprises at least one transcription termination site.

34. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 33, characterized in that the transcription termination site has a binding sequence of a mitochondrial transcription termination factor.

35. The chimerical peptide-nucleic acid fragment according to claim 34, characterized in that the transcription termination site has the binding sequence of a preferably bidirectionally acting transcription termination factor.

36. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 35, characterized in that the replication origin is a mitochondrial replication origin, preferably the replication origin of the heavy mtDNA strand having at least one 'conserved sequence block'.

37. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 36, characterized in that the plasmid further comprises at least one regulatory sequence for the replication.

38. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 37, characterized in that the regulatory sequence for the replication is a mitochondrial sequence motif.

39. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 38, characterized in that the plasmid further comprises a selection gene, preferably an antibiotic-resistance gene, preferably the oligomycin - or chloramphenicol - resistance gene.

40. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 39, characterized in that the plasmid further contains a multiple cloning site which permits the expression of 'foreign genes'.

41. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 40, characterized in that the multiple cloning site comprises recognition sequences for restriction endonucleases which do preferably not occur in another site of the plasmid.

42. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 41, characterized in that the multiple cloning site is arranged in the 3' direction of the promoter and in the 5' direction of the transcription termination site.

43. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 42, characterized in that the multiple cloning site is arranged in the 5' direction of the selection gene.

44. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 43, characterized in that the nucleic acid fragment has (phosphorylated) ends capable of litigation.

45. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 44, characterized in that the nucleic acid fragment has 'blunt ends' or overhanging 3' ends, preferably overhanging 5' ends.

46. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 45, characterized in that the nucleic acid fragment has 4 nucleotides comprising 5' overhangs which do not have a self-homology (palindromic sequence) and are not complementary to one another either.

47. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 46, characterized in that the ends of the nucleic acid fragment are cyclized via synthetic oligonucleotides.

48. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 47, characterized in that the overhanging 5' ends of the two oligonucleotides are complementary to one differing end of the nucleic acid each.

49. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 48, characterized in that two differing 'hairpin loops' are used for the cyclization, one being specific (complementary) to the 'left' plasmid end and the other being specific to the 'right' plasmid end of the nucleic acid.

50. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 49, characterized in that the modified nucleotide is localized preferably within the 'loop'.

51. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 50, characterized in that the plasmid DNA is amplified enzymatically by suitable oligonucleotides which have at least one recognition sequence for a restriction endonuclease which occurs preferably in non-repeated fashion in the plasmid sequence.

52. The chimerical peptide-nucleic acid fragment according to claim 51, characterized in that the restriction endonuclease to be used generated overhanging ends, preferably 5' overhanging ends, the cleavage site being localized preferably outside the recognition sequence.

53. The chimerical peptide-nucleic acid fragment according to claim 51 or 52, characterized in that the restriction endonuclease is B*sa*I.

54. A process for the production of a chimerical peptide-nucleic acid fragment according to any one of claims 1 to 53, characterized by the following stages:

(a) reaction of a nucleic acid (oligonucleotide) containing a functional linkage group having a linkage agent,

(b) reaction of the construct of (a) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL signal sequence, and

(c) optionally extension of the chimerical peptide-nucleic acid fragment of (b) by further DNA or RNA fragments.

55. The process according to claim 54, characterized in that the DNA in step (c) is a PCR-amplified DNA fragment containing the human mitochondrial promoter of the light strand (P_L) as well as the gene for the mitochondrial transfer RNA leucine ($tRNALeu^{UUR)}$).

56. The process for the production of a chimerical peptide-nucleic acid fragment according to any one of claims 1 to 53, characterized by the following steps:

(a) optional extension of the nucleic acid containing a functional linkage group by further DNA or RNA fragments,

(b) reaction of the nucleic acid with functional linkage group or the extended nucleic acid of (a) with a linkage agent,

(c) reaction of the construct of (b) with amino acids at the carboxy-terminal end of a peptide containing a signal sequence, with the exception of a KDEL signal sequence.

57. The process according to claim 56, characterized in that the DNA in step (a) is a PCR-amplified DNA fragment containing the human mitochondrial promoter of the light strand (P_L) as well as the gene for the mitochondrial transfer RNA leucine ($tRNALeu^{UUR)}$).

58. Use of the chimerical peptide-nucleic acid fragment according to any one of claims 1 to 53 for the appropriate nucleic acid introduction into cell organelles and cells, characterized by reacting the fragment with cells or pretreated cell compartments.

59. Use according to claim 58, characterized in that the pretreated cell compartments are energized mitochondria.

60. Use of the chimerical peptide-nucleic acid fragment according to any one of claims 1 to 59 for the introduction into eukaryotic cells.

61. Use of a chimerical peptide-nucleic acid fragment according to claim 60, characterized by employing the 'particle gun' system, electroporation, microinjection or lipotransfection for the introduction into eukaryotic cells.

ABSTRACT

This invention relates to a chimerical peptide-nucleic acid fragment, the process for producing the same and its use for appropriately introducing nucleic acids into cell organelles and cells.

Figure 1

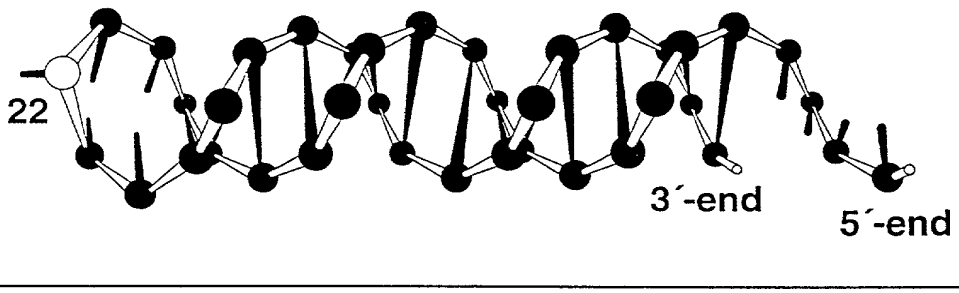
M-L-S-N-L-R-I-L-L-N-K-A-A-L-R-K-A-H-T-S-M-V-R-N-F-R-Y-G-K-P-V-Q-S-Q-L-K-P-R-D-L-C

amino-terminal end ⟷ carboxy-terminal end

22

CCCCGGGTACCTTGCGAGCCCTGGGCTCGCAAGGTACCC

5´-end ⟷ 3´-end



08765244 - 960524

08/765244

Figure 2

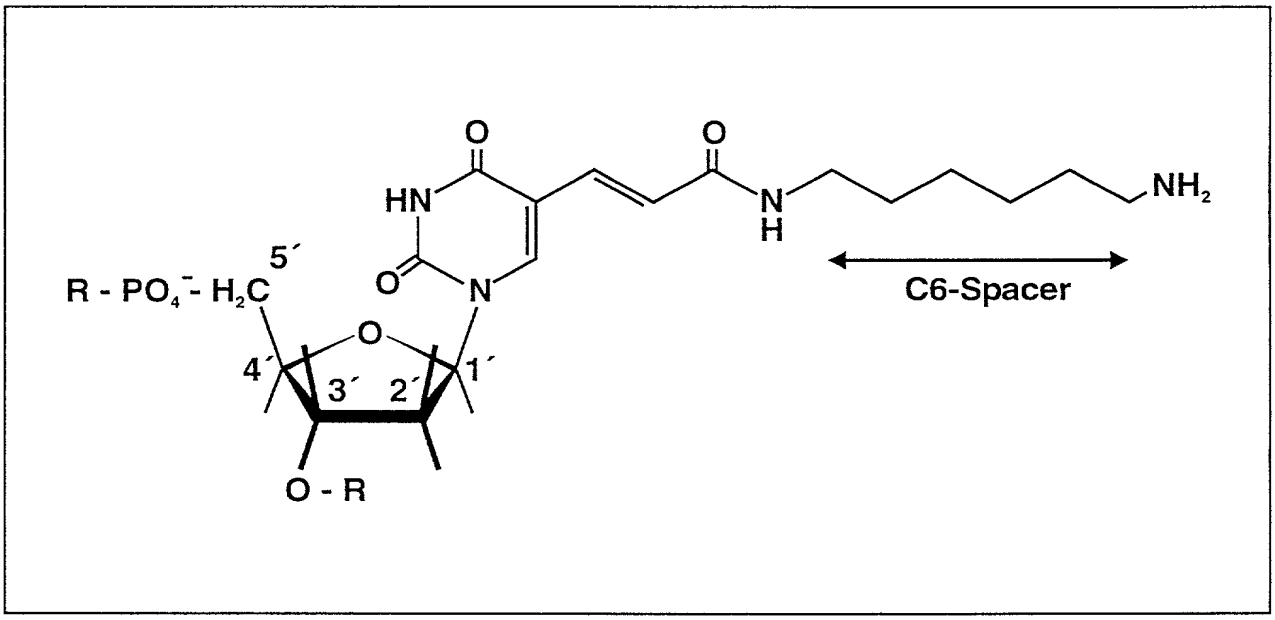


Figure 3

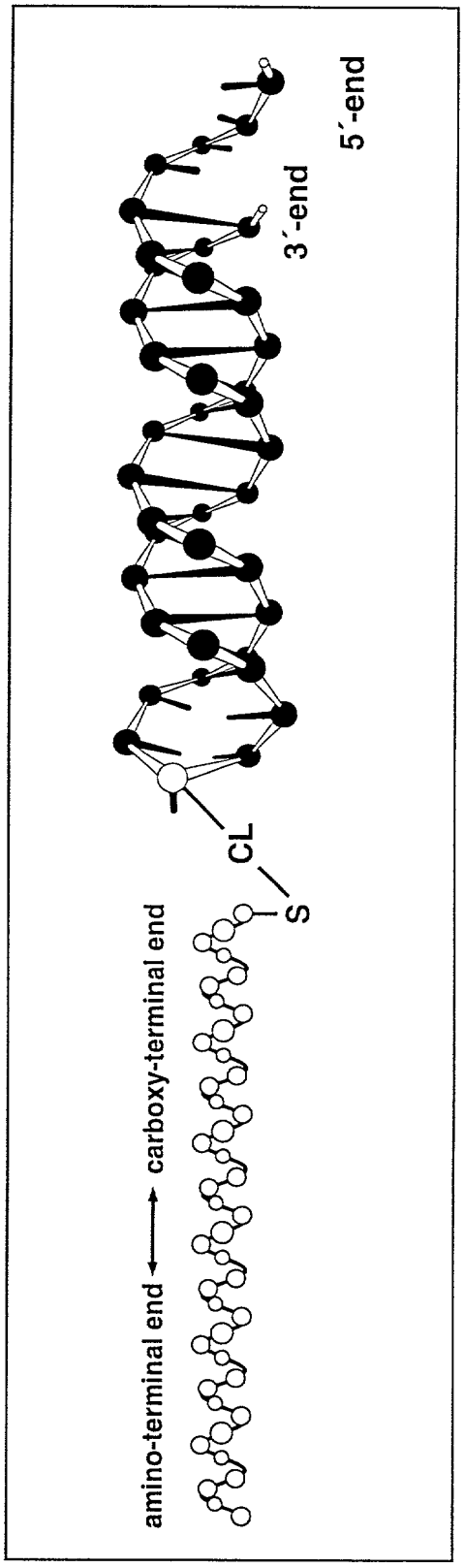


Figure 4

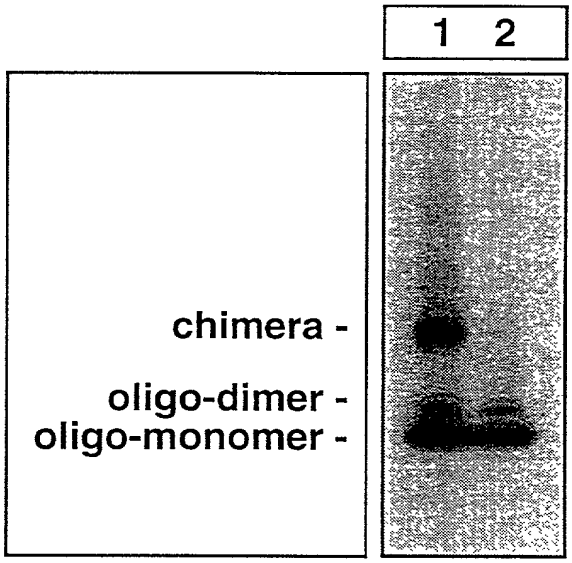


Figure 5a

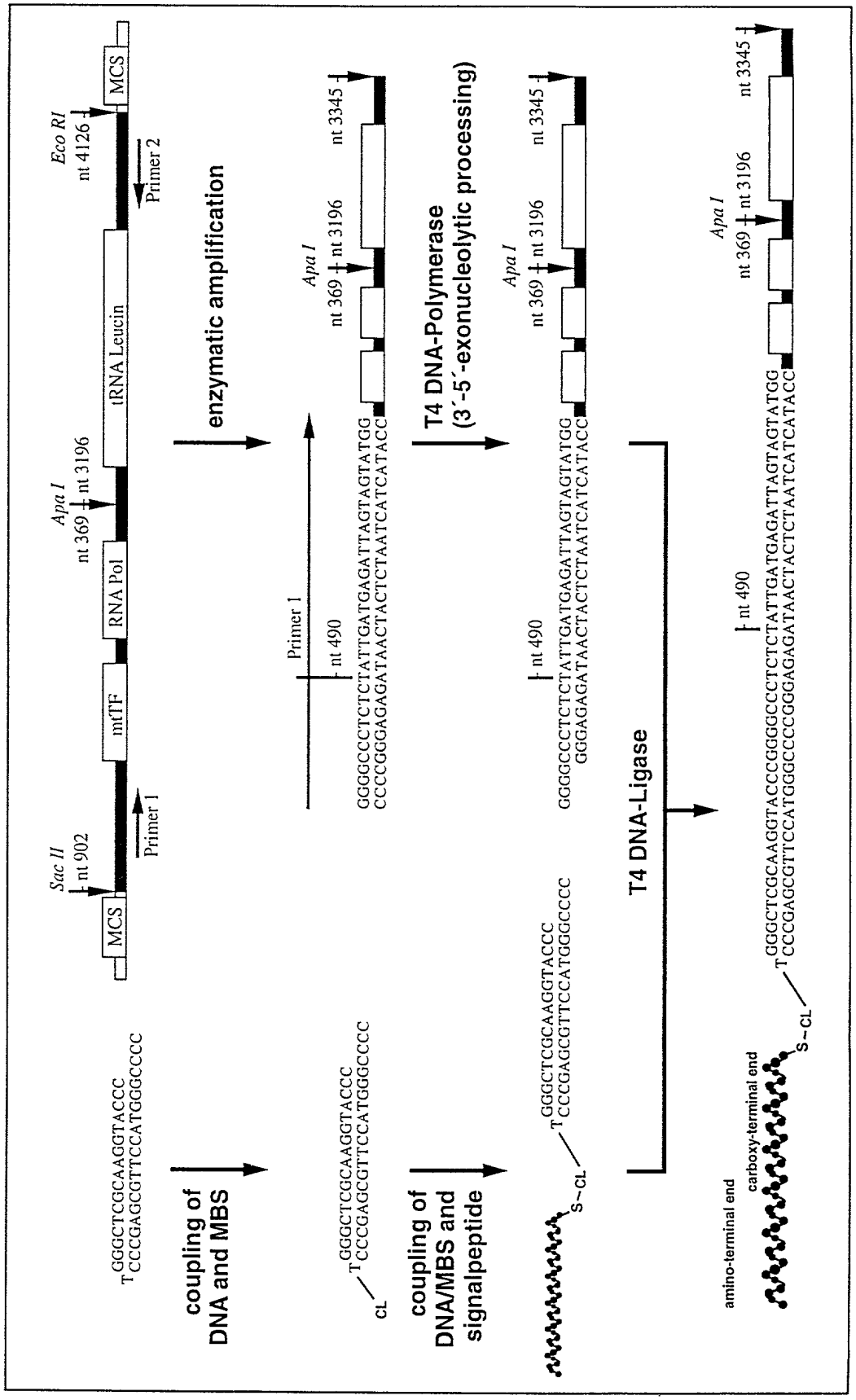

Sac II
nt 902
MCS
Primer 1
Apa I
nt 369
nt 3196
mtTF
RNA Pol
tRNA Leucin
Eco RI
nt 4126
MCS
Primer 2
GGGCTCGCAAGGTACCC
CCCGAGCGTTCCATGGGCCCC
coupling of DNA and MBS
enzymatic amplification
Primer 1
nt 490
Apa I
nt 369
nt 3196
nt 3345
GGGGCCCTCTCTATTGATGAGATTAGTAGTATGG
CCCCGGGAGAGATAACTACTCTAATCATCATACC
CL
coupling of DNA/MBS and signalpeptide
T4 DNA-Polymerase
(3´-5´-exonucleolytic processing)
S~CL
T4 DNA-Ligase
amino-terminal end
carboxy-terminal end

Figure 5b

```
        10          20          30          40          50          60
CCGCGGTGGC TGGCACGAAA TTGACCAACC CTGGGGTTAG TATAGCTTAG TAAACTTTC
GGCGCCACCG ACCGTGCTTT AACTGGTTGG GACCCCAATC ATATCGAATC ATTTGAAAG

        70          80          90          100         110         120
GTTTATTGCT AAAGGTTAAT CACTGCTGTT TCCCGTGGGG GTGTGGCTAG CTAAGCGTT
CAAATAACGA TTTCCAATTA GTGACGACAA AGGGCACCCC CACACCGATC GATTCGCAA

        130         140         150         160         170         180
TTGAGCTGCA TTGCTGCGTG CTTGATGCTT GTTCCTTTTG ATCGTGGTGA TTAGAGGGT
AACTCGACGT AACGACGCAC GAACTACGAA CAAGGAAAAC TAGCACCACT AATCTCCCA

        190         200         210         220         230         240
GAACTCACTG GAACGGGGAT GCTTGCATGT GTAATCTTAC TAAGAGCTAA AGAAAGGCT
CTTGAGTGAC CTTGCCCCTA CGAACGTACA CATTAGAATG ATTCTCGATT TCTTTCCGA

        250         260         270         280         290         300
AGGACCAAAC CTATTTGTTT ATGGGGTGAT GTGAGCCCGT CTAAACATTT CAGTGTATT
TCCTGGTTTG GATAAACAAA TACCCCACTA CACTCGGGCA GATTTGTAAA GTCACATAA

        310         320         330         340         350         360
GCTTTGAGGA GGTAAGCTAC ATAAACTGTG GGGGGTGTCT TTGGGGTTTG TTGGTTCGG
CGAAACTCCT CCATTCGATG TATTTGACAC CCCCCACAGA AACCCCAAAC AACCAAGCC

        370         380         390         400         410         420
GGTATGGGGT TAGCAGCGGT GTGTGTGTGC TGGGTAGGAT GGGCGGGGGT GTATTGATG
CCATACCCCA ATCGTCGCCA CACACACACG ACCCATCCTA CCCGCCCCCA CATAACTAC

        430         440         450         460         470         480
AGATTAGTAG TATGGGAGTG GGAGGGGAAA ATAATGTGTT AGTTGGGGGG GACTGTTAA
TCTAATCATC ATACCCTCAC CCTCCCCTTT TATTACACAA TCAACCCCCC CTGACAATT

        490         500         510         520         530         540
AAGTGCATAC CGCCAAAAGA TAAAATTTGA AATCTGGTTA GGCTGGTGTT GGGCCCTTT
TTCACGTATG GCGGTTTTCT ATTTTAAACT TTAGACCAAT CCGACCACAA CCCGGGAAA

        550         560         570         580         590         600
GTCCCACACC CACCCAAGAA CAGGGTTTGT TAAGATGGCA GAGCCCGGTA TCGCATAAA
CAGGGTGTGG GTGGGTTCTT GTCCCAAACA ATTCTACCGT CTCGGGCCAT AGCGTATTT

        610         620         630         640         650         660
ACTTAAAACT TTACAGTCAG AGGTTCAATT CCTCTTCTTA ACAACATACC ATGGCCAAC
TGAATTTTGA AATGTCAGTC TCCAAGTTAA GGAGAAGAAT TGTTGTATGG TACCGGTTG

        670         680         690         700         710         720
CTCCTACTCC TCATTGTACC CATTCTAATC GCAATGGCAT TCCTAATGCT ACCGAACGA
GAGGATGAGG AGTAACATGG GTAAGATTAG CGTTACCGTA AGGATTACGA TGGCTTGCT

        730         740         750         760         770         780
AAAATTCTAG GCTATATACA ACTACGCAAA GGCCCCAACG TGGTAGGCCC TACGGGCTA
TTTTAAGATC CGATATATGT TGATGCGTTT CCGGGGTTGC ACCATCCGGG ATGCCCGAT

        790         800         810         820         830         840
CTACAACCCT TCGCTGACGC CATAAAACTC TTCACCAAAG AGCCCCTAAA CCCGCCACA
GATGTTGGGA AGCGACTGCG GTATTTTGAG AAGTGGTTTC TCGGGGATTT GGGCGGTGT
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       850        860        870        880        890        900
TCTACCATCA CCCTCTACAT CACCGCCCCG ACCTTAGCTC TCACCATCGC CTTCTACTA
AGATGGTAGT GGGAGATGTA GTGGCGGGGC TGGAATCGAG AGTGGTAGCG GAAGATGAT

       910        920        930        940        950        960
TGAACCCCCC TCCCCATACC CAACCCCCTG GTCAACCTCA ACCTAGGCCT CTATTTATT
ACTTGGGGGG AGGGGTATGG GTTGGGGGAC CAGTTGGAGT TGGATCCGGA GATAAATAA

       970        980        990       1000       1010       1020
CTAGCCACCT CTAGCCTAGC CGTTTACTCA ATCCTCTGAT CAGGGTGAGC TCAAACTCA
GATCGGTGGA GATCGGATCG GCAAATGAGT TAGGAGACTA GTCCCACTCG AGTTTGAGT

      1030       1040       1050       1060       1070       1080
AACTACGCCC TGATCGGCGC ACTGCGAGCA GTAGCCCAAA CAATCTCATA GAAGTCACC
TTGATGCGGG ACTAGCCGCG TGACGCTCGT CATCGGGTTT GTTAGAGTAT CTTCAGTGG

      1090       1100       1110       1120       1130       1140
CTAGCCATCA TTCTACTATC AACATTACTA ATAAGTGGCT CCTTTAACCT TCCACCCTT
GATCGGTAGT AAGATGATAG TTGTAATGAT TATTCACCGA GGAAATTGGA AGGTGGGAA

      1150       1160       1170       1180       1190       1200
ATCACAACAC AAGAACACCT CTGATTACTC CTGCCATCAT GACCCTTGGC ATAATATGA
TAGTGTTGTG TTCTTGTGGA GACTAATGAG GACGGTAGTA CTGGGAACCG TATTATACT

      1210       1220       1230       1240       1250       1260
TTTATCTCCA CACTAGCAGA GACCAACCGA ACCCCCTTCG ACCTTGCCGA GGGGAGTCC
AAATAGAGGT GTGATCGTCT CTGGTTGGCT TGGGGGAAGC TGGAACGGCT CCCCTCAGG

      1270       1280       1290       1300       1310       1320
GAACTAGTCT CAGGCTTCAA CATCGAATAC GCCGCAGGCC CCTTCGCCCT TTCTTCATA
CTTGATCAGA GTCCGAAGTT GTAGCTTATG CGGCGTCCGG GGAAGCGGGA AAGAAGTAT

      1330       1340       1350       1360       1370       1380
GCCGAATACA CAAACATTAT TATAATAAAC ACCCTCACCA CTACAATCTT CTAGGAACA
CGGCTTATGT GTTTGTAATA ATATTATTTG TGGGAGTGGT GATGTTAGAA GATCCTTGT

      1390       1400       1410       1420       1430       1440
ACATATGACG CACTCTCCCC TGAACTCTAC ACAACATATT TTGTCACCAA ACCCTACTT
TGTATACTGC GTGAGAGGGG ACTTGAGATG TGTTGTATAA AACAGTGGTT TGGGATGAA

      1450       1460
CTAACCTCCC TGTTCTTATG AATTC
GATTGGAGGG ACAAGAATAC TTAAG
```

Figure 6a

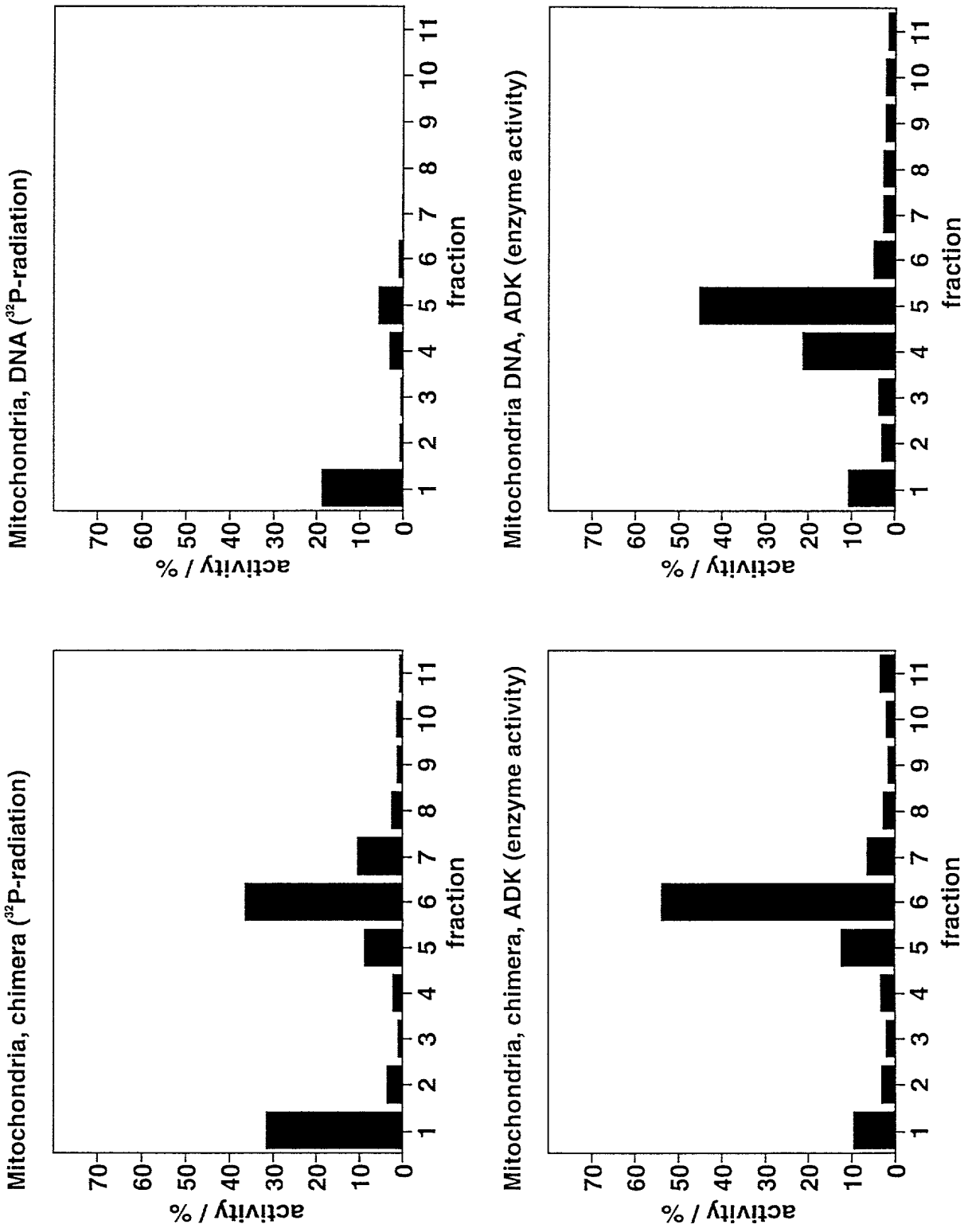
Mitochondria, DNA (^{32}P-radiation)
Mitochondria DNA, ADK (enzyme activity)
Mitochondria, chimera (^{32}P-radiation)
Mitochondria, chimera, ADK (enzyme activity)
activity / %
fraction
0
10
20
30
40
50
60
70
1
2
3
4
5
6
7
8
9
10
11

Figure 6b

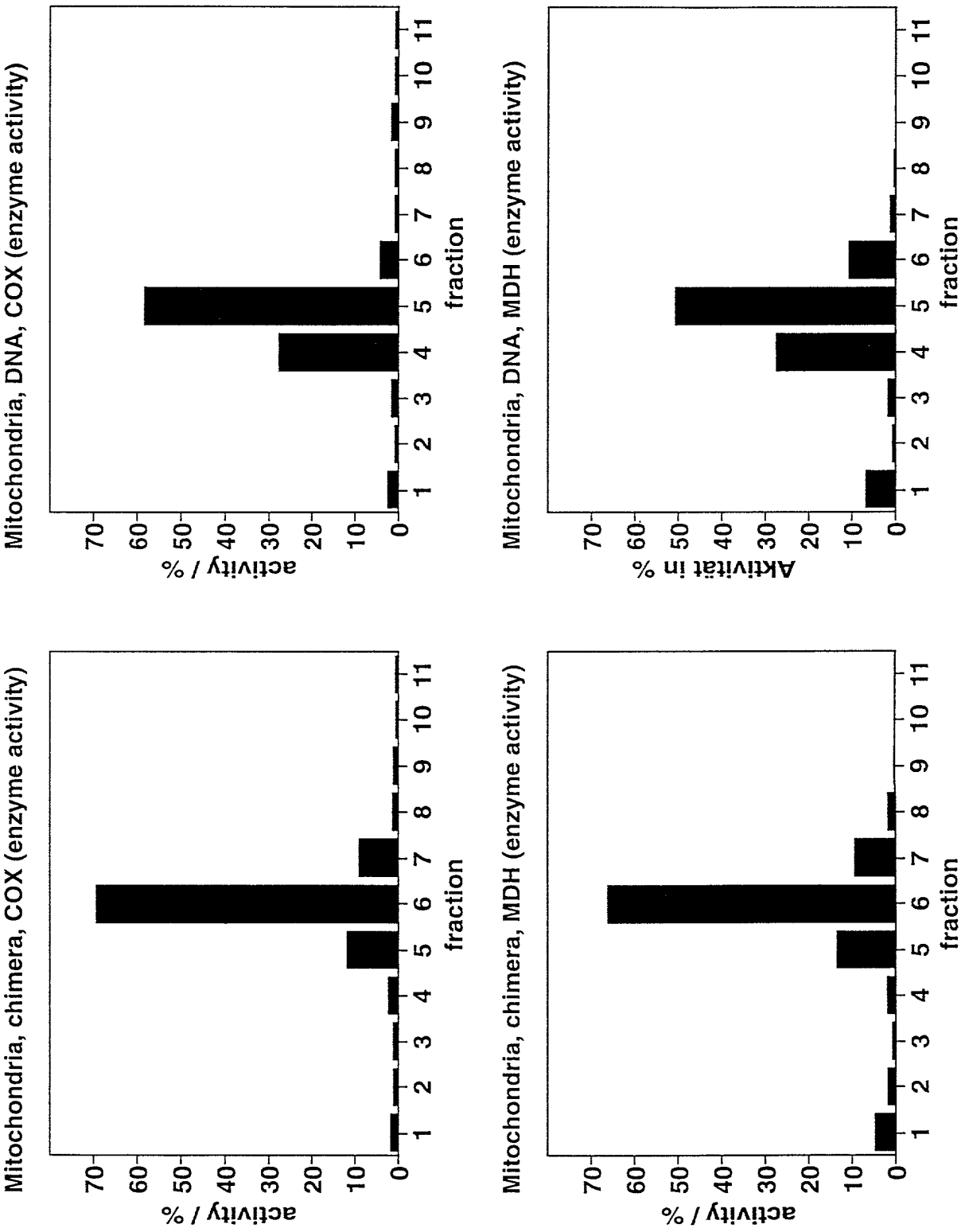
Mitochondria, DNA, COX (enzyme activity)
activity / %
fraction
Mitochondria, DNA, MDH (enzyme activity)
Aktivität in %
fraction
Mitochondria, chimera, COX (enzyme activity)
activity / %
fraction
Mitochondria, chimera, MDH (enzyme activity)
activity / %
fraction

Figure 7a

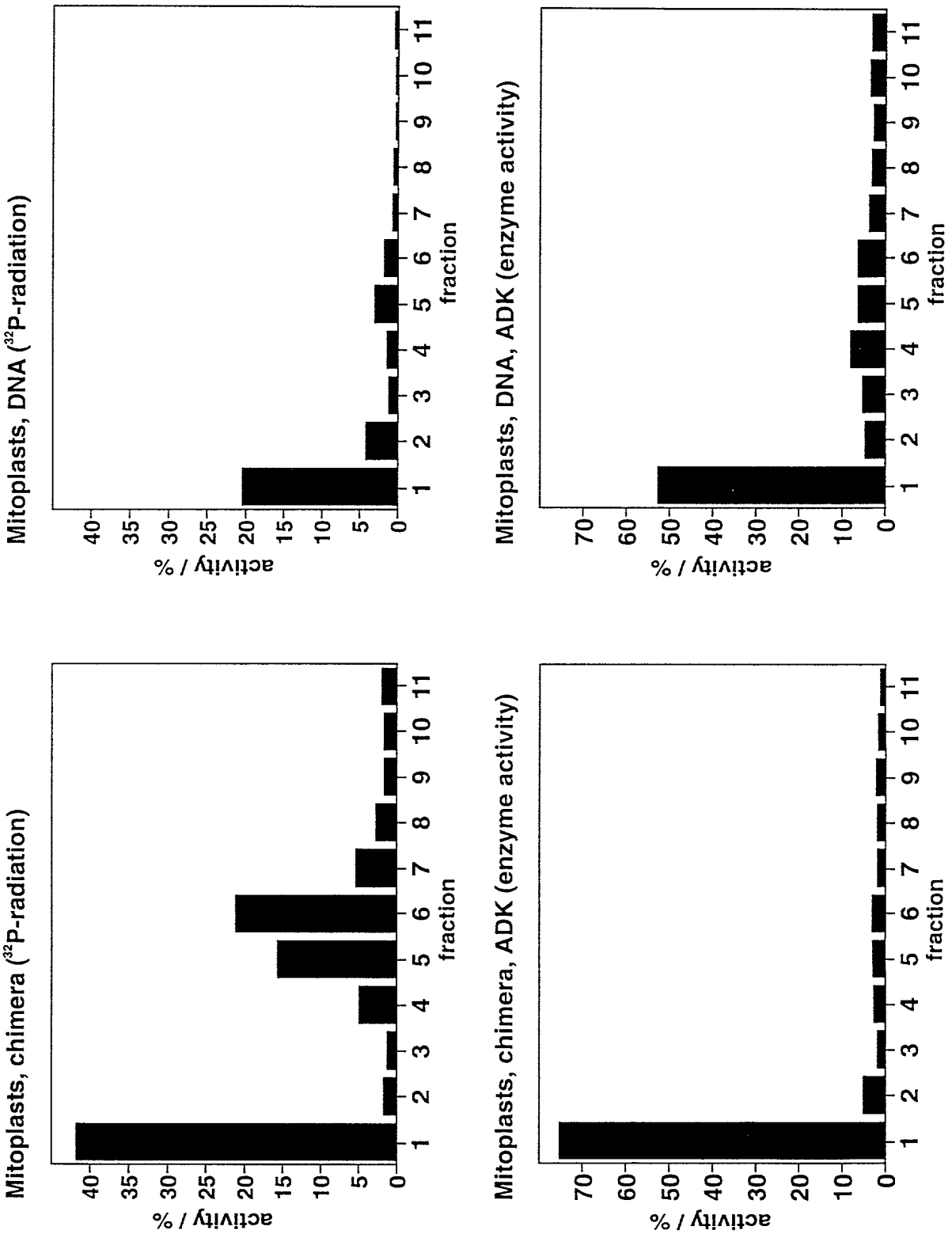
Mitoplasts, DNA (^{32}P-radiation)
Mitoplasts, DNA, ADK (enzyme activity)
Mitoplasts, chimera (^{32}P-radiation)
Mitoplasts, chimera, ADK (enzyme activity)
activity / %
fraction
0
5
10
15
20
25
30
35
40
10
20
30
40
50
60
70
1
2
3
4
5
6
7
8
9
10
11

Figure 7b

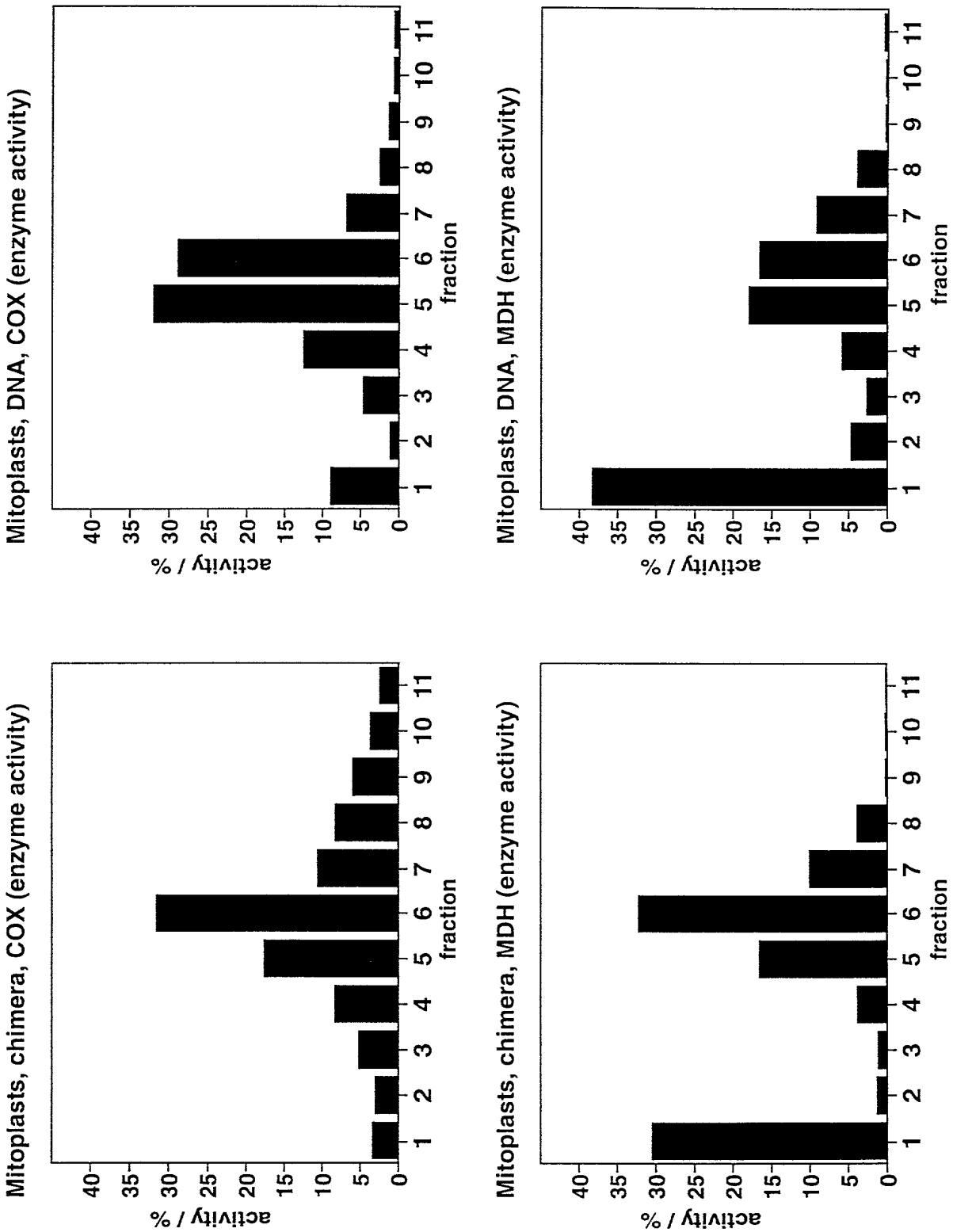
Mitoplasts, DNA, COX (enzyme activity)
Mitoplasts, DNA, MDH (enzyme activity)
Mitoplasts, chimera, COX (enzyme activity)
Mitoplasts, chimera, MDH (enzyme activity)
activity / %
fraction
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6
7
8
9
10
11

Figure 8

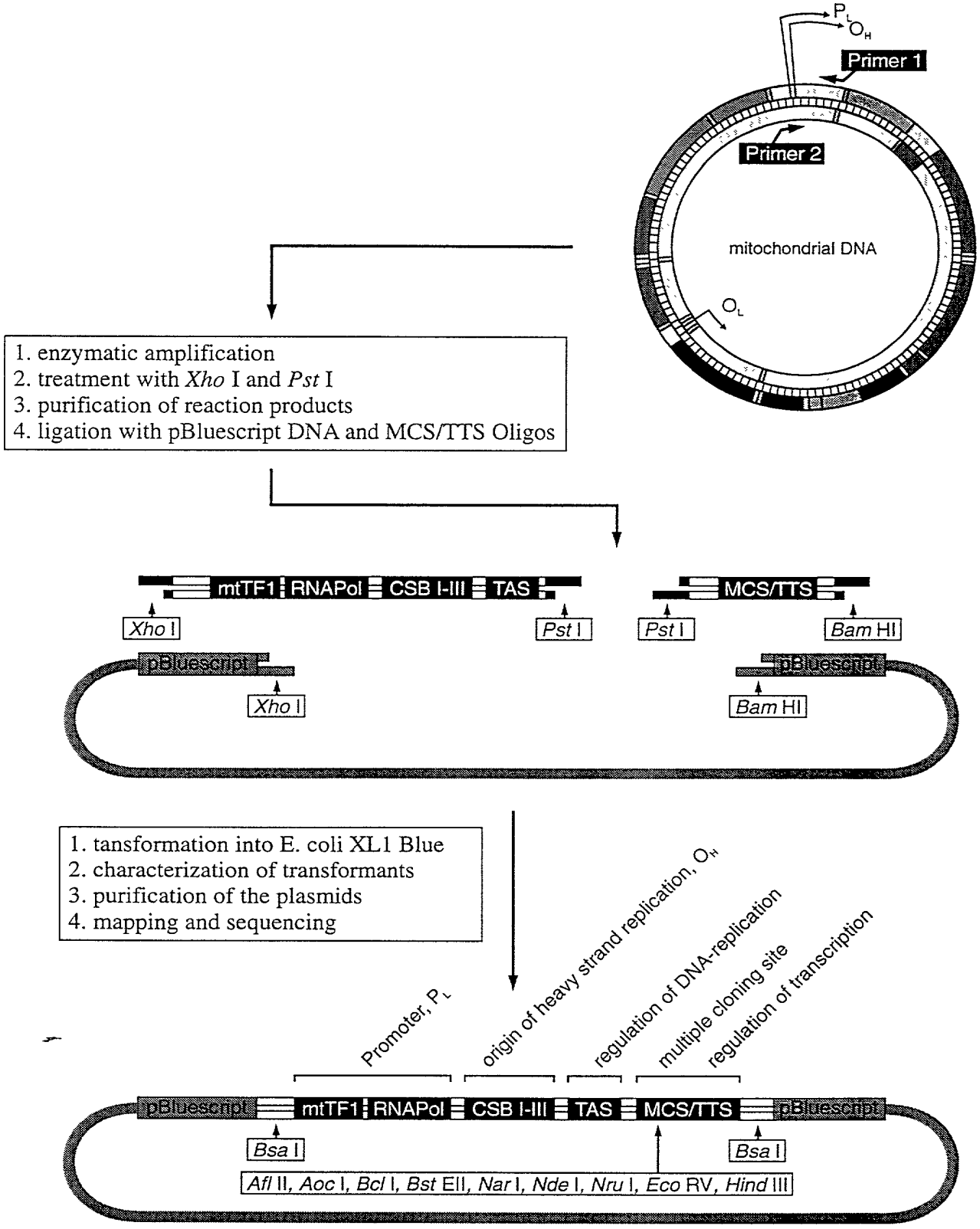
P_L
O_H
Primer 1
Primer 2
mitochondrial DNA
O_L
1. enzymatic amplification
2. treatment with Xho I and Pst I
3. purification of reaction products
4. ligation with pBluescript DNA and MCS/TTS Oligos
mtTF1
RNAPol
CSB I-III
TAS
MCS/TTS
Xho I
Pst I
Pst I
Bam HI
pBluescript
Xho I
pBluescript
Bam HI
1. tansformation into E. coli XL1 Blue
2. characterization of transformants
3. purification of the plasmids
4. mapping and sequencing
Promoter, P_L
origin of heavy strand replication, O_H
regulation of DNA-replication
multiple cloning site
regulation of transcription
pBluescript
mtTF1
RNAPol
CSB I-III
TAS
MCS/TTS
pBluescript
Bsa I
Bsa I
Afl II, Aoc I, Bcl I, Bst EII, Nar I, Nde I, Nru I, Eco RV, Hind III

Figure 9

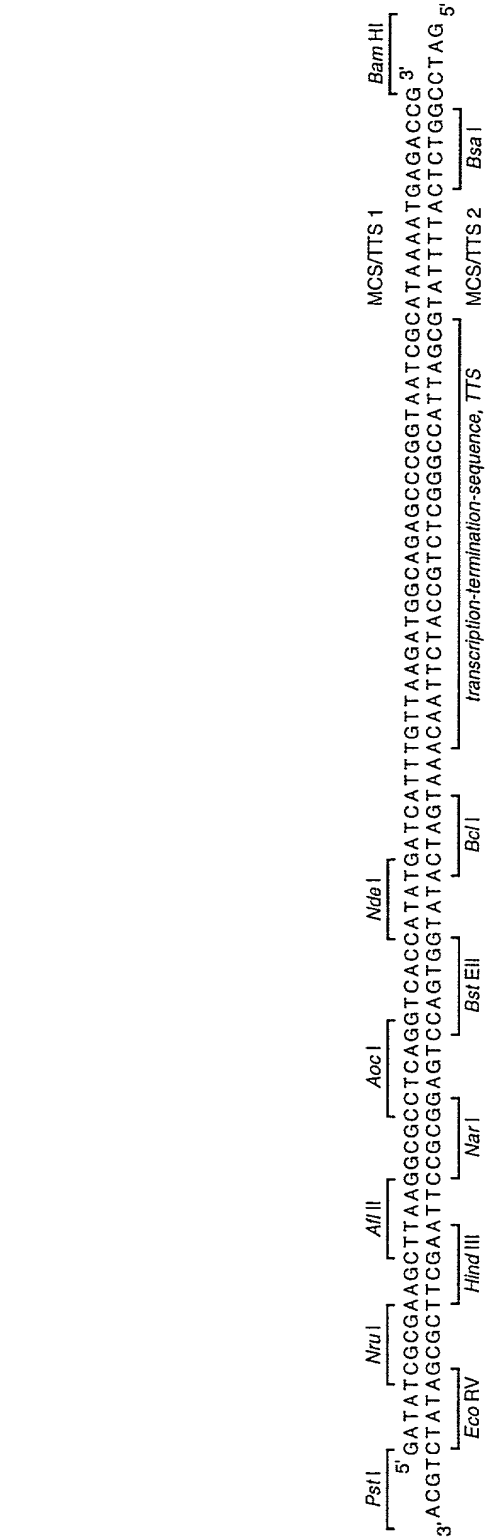
Pst I
Eco RV
Ntru I
Hind III
Afl II
Nar I
Aoc I
Bst EII
Nde I
Bcl I
transcription-termination-sequence, TTS
MCS/TTS 2
MCS/TTS 1
Bsa I
Bam HI
5' GATATCGCGAAGCTTAAGGCGCCTCAGGTCACCATATGATCATTTGTTAAGATGGCAGAGCCCGGTAATCGCATAAAATGAGACCG 3'
3' ACGTCTATAGCGCTTCGAATTCCGCGGAGTCCAGTGGTATACTAGTAAACAATTCTACCGTCTCGGGCCATTAGCGTATTTTACTCTGGCCTAG 5'

Figure 10


```
        10          20          30          40          50          60
CTCGAGGGTC TCAGGGGCTA ATAGAAAGGC TAGGACCAAA CCTATTTGTT TATGGGGTGA
GAGCTCCCAG AGTCCCCGAT TATCTTTCCG ATCCTGGTTT GGATAAACAA ATACCCCACT

        70          80          90          100         110         120
TGTGAGCCCG TCTAAACATT TTCAGTGTAT TGCTTTGAGG AGGTAAGCTA CATAAACTGT
ACACTCGGGC AGATTTGTAA AAGTCACATA ACGAAACTCC TCCATTCGAT GTATTTGACA

        130         140         150         160         170         180
GGGGGGTGTC TTTGGGGTTT GGTTGGTTCG GGGTATGGGG TTAGCAGCGG TGTGTGTGTG
CCCCCCACAG AAACCCCAAA CCAACCAAGC CCCATACCCC AATCGTCGCC ACACACACAC

        190         200         210         220         230         240
CTGGGTAGGA TGGGCGGGGG TTGTATTGAT GAGATTAGTA GTATGGGAGT GGGAGGGGAA
GACCCATCCT ACCCGCCCCC AACATAACTA CTCTAATCAT CATACCCTCA CCCTCCCCTT

        250         260         270         280         290         300
AATAATGTGT TAGTTGGGGG GTGACTGTTA AAAGTGCATA CCGCCAAAAG ATAAAATTTG
TTATTACACA ATCAACCCCC CACTGACAAT TTTCACGTAT GGCGGTTTTC TATTTTAAAC

        310         320         330         340         350         360
AAATCTGGTT AGGCTGGTGT TAGGGTTCTT TGTTTTTGGG GTTTGGCAGA GATGTGTTTA
TTTAGACCAA TCCGACCACA ATCCCAAGAA ACAAAAACCC CAAACCGTCT CTACACAAAT

        370         380         390         400         410         420
AGTGCTGTGG CCAGAAGCGG GGGAGGGGGG GTTTGGTGGA AATTTTTTGT TATGATGTCT
TCACGACACC GGTCTTCGCC CCCTCCCCCC CAAACCACCT TTAAAAAACA ATACTACAGA

        430         440         450         460         470         480
GTGTGGAAAG TGGCTGTGCA GACATTCAAT TGTTATTATT ATGTCCTACA AGCATTAATT
CACACCTTTC ACCGACACGT CTGTAAGTTA ACAATAATAA TACAGGATGT TCGTAATTAA

        490         500         510         520         530         540
AATTAACACA CTTTAGTAAG TATGTTCGCC TGTAATATTG AACGTAGGTG CGATAAATAA
TTAATTGTGT GAAATCATTC ATACAAGCGG ACATTATAAC TTGCATCCAC GCTATTTATT

        550         560         570         580         590         600
TAGGATGAGG CAGGAATCAA AGACAGATAC TGCGACATAG GGTGCTCCGG CTCCAGCGTC
ATCCTACTCC GTCCTTAGTT TCTGTCTATG ACGCTGTATC CCACGAGGCC GAGGTCGCAG

        610         620         630         640         650         660
TCGCAATGCT ATCGCGTGCA TACCCCCCAG ACGAAAATAC CAAATGCATG GAGAGCTCCC
AGCGTTACGA TAGCGCACGT ATGGGGGGTC TGCTTTTATG GTTTACGTAC CTCTCGAGGG

        670         680         690         700         710         720
GTGAGTGGTT AATAGGGTGA TAGACCTGTG ATCCATCGTG ATGTCTTATT TAAGGGGAAC
CACTCACCAA TTATCCCACT ATCTGGACAC TAGGTAGCAC TACAGAATAA ATTCCCCTTG

        730         740         750         760         770         780
GTGTGGGCTA TTTAGGCTTT ATGACCCTGA AGTAGGAACC AGATGTCGGA TACAGTTCAC
CACACCCGAT AAATCCGAAA TACTGGGACT TCATCCTTGG TCTACAGCCT ATGTCAAGTG
```

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       790         800         810         820         830         840
TTTAGCTACC CCCAAGTGTT ATGGGCCCGG AGCGAGGAGA GTAGCACTCT TGTGCGGGAT
AAATCGATGG GGGTTCACAA TACCCGGGCC TCGCTCCTCT CATCGTGAGA ACACGCCCTA

       850         860         870         880         890         900
ATTGATTTCA CGGAGGATGG TGGTCAAGGG ACCCCTATCT GAGGGGGGTC ATCCATGGGG
TAACTAAAGT GCCTCCTACC ACCAGTTCCC TGGGGATAGA CTCCCCCCAG TAGGTACCCC

       910         920         930         940         950         960
ACGAGAAGGG ATTTGACTGT AATGTGCTAT GTACGGTAAA TGGCTTTATG TACTATGTAC
TGCTCTTCCC TAAACTGACA TTACACGATA CATGCCATTT ACCGAAATAC ATGATACATG

       970         980         990        1000        1010        1020
TGTTAAGGGT GGGTAGGTTT GTTGGTATCC TAGTGGGTGA GGGGTGGCTT TGGAGTTGCA
ACAATTCCCA CCCATCCAAA CAACCATAGG ATCACCCACT CCCCACCGAA ACCTCAACGT

      1030        1040        1050        1060        1070        1080
GTTGATGTGT GATAGTTGAG GGTTGATTGC TGTACTTGCT TGTAAGCATG GGGAGGGGGT
CAACTACACA CTATCAACTC CCAACTAACG ACATGAACGA ACATTCGTAC CCCTCCCCCA

      1090        1100        1110        1120        1130        1140
TTTGATGTGG ATTGGGTTTT TATGTACTAC AGGTGGTCAA GTATTTATGG TACCGTACAA
AAACTACACC TAACCCAAAA ATACATGATG TCCACCAGTT CATAAATACC ATGGCATGTT

      1150        1160        1170        1180        1190        1200
TATTCATGGT GGCTGGCAGT AATGTACGAA ATACATAGCG GTTGTTGATG GGTGAGTCAA
ATAAGTACCA CCGACCGTCA TTACATGCTT TATGTATCGC CAACAACTAC CCACTCAGTT

      1210        1220        1230        1240        1250        1260
TACTTGGGTG GTACCCAAAT CTGCTTCCCC ATGAAAGAAC AGAGAATAGT TTAAATTAGA
ATGAACCCAC CATGGGTTTA GACGAAGGGG TACTTTCTTG TCTCTTATCA AATTTAATCT

      1270        1280        1290        1300        1310        1320
ATCTTAGCTT TGGGTGCTAA TGGTGGAGTT AAAGACTTTT TCTCTGATTT GTCCTTGGAA
TAGAATCGAA ACCCACGATT ACCACCTCAA TTTCTGAAAA AGAGACTAAA CAGGAACCTT

      1330        1340        1350        1360        1370        1380
AAAGGTTTTC ATCTCCGGTT TACAAGACTG GTGTATTAGC TGCAGATATC GCGAAGCTTA
TTTCCAAAAG TAGAGGCCAA ATGTTCTGAC CACATAATCG ACGTCTATAG CGCTTCGAAT

      1390        1400        1410        1420        1430        1440
AGGCGCCTCA GGTCACCATA TGATCATTTG TTAAGATGGC AGAGCCCGGT AATCGCATAA
TCCGCGGAGT CCAGTGGTAT ACTAGTAAAC AATTCTACCG TCTCGGGCCA TTAGCGTATT

      1450
AATGAGACCG GATCC
TTACTCTGGC CTAGG
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SUBSTITUTE SHEET (RULE 26)

Figure 11

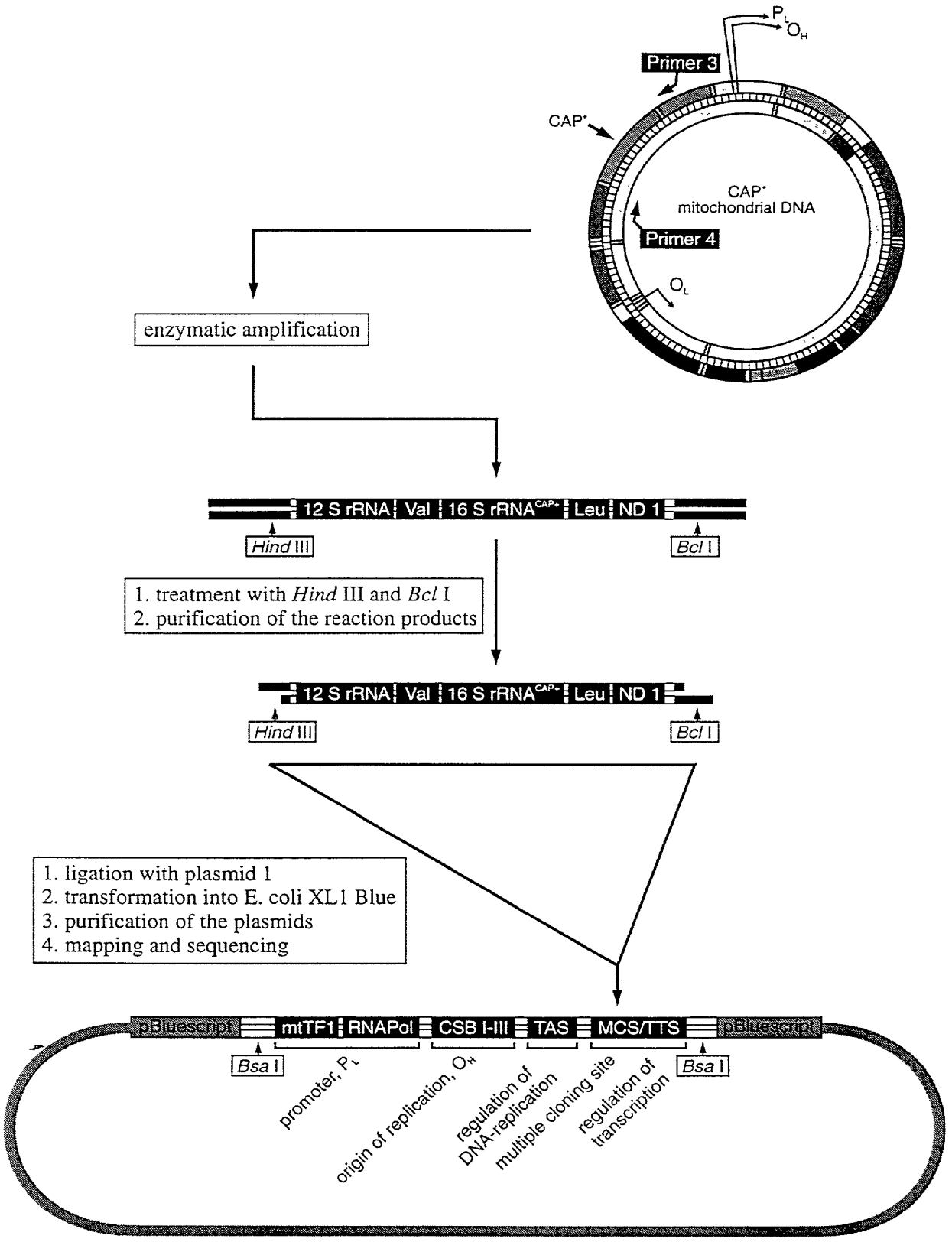
P_L
O_H
Primer 3
CAP+
CAP+ mitochondrial DNA
Primer 4
O_L
enzymatic amplification
12 S rRNA | Val | 16 S rRNA CAP+ | Leu | ND 1
Hind III
Bcl I
1. treatment with Hind III and Bcl I
2. purification of the reaction products
12 S rRNA | Val | 16 S rRNA CAP+ | Leu | ND 1
Hind III
Bcl I
1. ligation with plasmid 1
2. transformation into E. coli XL1 Blue
3. purification of the plasmids
4. mapping and sequencing
pBluescript
mtTF1 | RNAPol | CSB I-III | TAS | MCS/TTS
pBluescript
Bsa I
promoter, P_L
origin of replication, O_H
regulation of DNA-replication
multiple cloning site
regulation of transcription
Bsa I

Figure 12


```
        10          20          30          40          50          60
CTCGAGGGTC TCAGGGGCTA ATAGAAAGGC TAGGACCAAA CCTATTTGTT TATGGGGTGA
GAGCTCCCAG AGTCCCCGAT TATCTTTCCG ATCCTGGTTT GGATAAACAA ATACCCCACT

        70          80          90          100         110         120
TGTGAGCCCG TCTAAACATT TTCAGTGTAT TGCTTTGAGG AGGTAAGCTA CATAAACTGT
ACACTCGGGC AGATTTGTAA AAGTCACATA ACGAAACTCC TCCATTCGAT GTATTTGACA

        130         140         150         160         170         180
GGGGGGTGTC TTTGGGGTTT GGTTGGTTCG GGGTATGGGG TTAGCAGCGG TGTGTGTGTG
CCCCCCACAG AAACCCCAAA CCAACCAAGC CCCATACCCC AATCGTCGCC ACACACACAC

        190         200         210         220         230         240
CTGGGTAGGA TGGGCGGGGG TTGTATTGAT GAGATTAGTA GTATGGGAGT GGGAGGGGAA
GACCCATCCT ACCCGCCCCC AACATAACTA CTCTAATCAT CATACCCTCA CCCTCCCCTT

        250         260         270         280         290         300
AATAATGTGT TAGTTGGGGG GTGACTGTTA AAAGTGCATA CCGCCAAAAG ATAAAATTTG
TTATTACACA ATCAACCCCC CACTGACAAT TTTCACGTAT GGCGGTTTTC TATTTTAAAC

        310         320         330         340         350         360
AAATCTGGTT AGGCTGGTGT TAGGGTTCTT TGTTTTTGGG GTTTGGCAGA GATGTGTTTA
TTTAGACCAA TCCGACCACA ATCCCAAGAA ACAAAAACCC CAAACCGTCT CTACACAAAT

        370         380         390         400         410         420
AGTGCTGTGG CCAGAAGCGG GGGAGGGGGG GTTTGGTGGA AATTTTTTGT TATGATGTCT
TCACGACACC GGTCTTCGCC CCCTCCCCCC CAAACCACCT TTAAAAAACA ATACTACAGA

        430         440         450         460         470         480
GTGTGGAAAG TGGCTGTGCA GACATTCAAT TGTTATTATT ATGTCCTACA AGCATTAATT
CACACCTTTC ACCGACACGT CTGTAAGTTA ACAATAATAA TACAGGATGT TCGTAATTAA

        490         500         510         520         530         540
AATTAACACA CTTTAGTAAG TATGTTCGCC TGTAATATTG AACGTAGGTG CGATAAATAA
TTAATTGTGT GAAATCATTC ATACAAGCGG ACATTATAAC TTGCATCCAC GCTATTTATT

        550         560         570         580         590         600
TAGGATGAGG CAGGAATCAA AGACAGATAC TGCGACATAG GGTGCTCCGG CTCCAGCGTC
ATCCTACTCC GTCCTTAGTT TCTGTCTATG ACGCTGTATC CCACGAGGCC GAGGTCGCAG

        610         620         630         640         650         660
TCGCAATGCT ATCGCGTGCA TACCCCCCAG ACGAAAATAC CAAATGCATG GAGAGCTCCC
AGCGTTACGA TAGCGCACGT ATGGGGGGTC TGCTTTTATG GTTTACGTAC CTCTCGAGGG

        670         680         690         700         710         720
GTGAGTGGTT AATAGGGTGA TAGACCTGTG ATCCATCGTG ATGTCTTATT TAAGGGGAAC
CACTCACCAA TTATCCCACT ATCTGGACAC TAGGTAGCAC TACAGAATAA ATTCCCCTTG

        730         740         750         760         770         780
GTGTGGGCTA TTTAGGCTTT ATGACCCTGA AGTAGGAACC AGATGTCGGA TACAGTTCAC
CACACCCGAT AAATCCGAAA TACTGGGACT TCATCCTTGG TCTACAGCCT ATGTCAAGTG

        790         800         810         820         830         840
TTTAGCTACC CCCAAGTGTT ATGGGCCCGG AGCGAGGAGA GTAGCACTCT TGTGCGGGAT
AAATCGATGG GGGTTCACAA TACCCGGGCC TCGCTCCTCT CATCGTGAGA ACACGCCCTA
```

```
       850         860         870         880         890         900
ATTGATTTCA CGGAGGATGG TGGTCAAGGG ACCCCTATCT GAGGGGGGTC ATCCATGGGG
TAACTAAAGT GCCTCCTACC ACCAGTTCCC TGGGGATAGA CTCCCCCCAG TAGGTACCCC

       910         920         930         940         950         960
ACGAGAAGGG ATTTGACTGT AATGTGCTAT GTACGGTAAA TGGCTTTATG TACTATGTAC
TGCTCTTCCC TAAACTGACA TTACACGATA CATGCCATTT ACCGAAATAC ATGATACATG

       970         980         990        1000        1010        1020
TGTTAAGGGT GGGTAGGTTT GTTGGTATCC TAGTGGGTGA GGGGTGGCTT TGGAGTTGCA
ACAATTCCCA CCCATCCAAA CAACCATAGG ATCACCCACT CCCCACCGAA ACCTCAACGT

      1030        1040        1050        1060        1070        1080
GTTGATGTGT GATAGTTGAG GGTTGATTGC TGTACTTGCT TGTAAGCATG GGGAGGGGGT
CAACTACACA CTATCAACTC CCAACTAACG ACATGAACGA ACATTCGTAC CCCTCCCCCA

      1090        1100        1110        1120        1130        1140
TTTGATGTGG ATTGGGTTTT TATGTACTAC AGGTGGTCAA GTATTTATGG TACCGTACAA
AAACTACACC TAACCCAAAA ATACATGATG TCCACCAGTT CATAAATACC ATGGCATGTT

      1150        1160        1170        1180        1190        1200
TATTCATGGT GGCTGGCAGT AATGTACGAA ATACATAGCG GTTGTTGATG GGTGAGTCAA
ATAAGTACCA CCGACCGTCA TTACATGCTT TATGTATCGC CAACAACTAC CCACTCAGTT

      1210        1220        1230        1240        1250        1260
TACTTGGGTG GTACCCAAAT CTGCTTCCCC ATGAAAGAAC AGAGAATAGT TTAAATTAGA
ATGAACCCAC CATGGGTTTA GACGAAGGGG TACTTTCTTG TCTCTTATCA AATTTAATCT

      1270        1280        1290        1300        1310        1320
ATCTTAGCTT TGGGTGCTAA TGGTGGAGTT AAAGACTTTT TCTCTGATTT GTCCTTGGAA
TAGAATCGAA ACCCACGATT ACCACCTCAA TTTCTGAAAA AGAGACTAAA CAGGAACCTT

      1330        1340        1350        1360        1370        1380
AAAGGTTTTC ATCTCCGGTT TACAAGACTG GTGTATTAGC TGCAGATATC GCGAAGCTTG
TTTCCAAAAG TAGAGGCCAA ATGTTCTGAC CACATAATCG ACGTCTATAG CGCTTCGAAC

      1390        1400        1410        1420        1430        1440
TAACATGGTA AGTGTACTGG AAAGTGCACT TGGACGAACC AGAGTGTAGC TTAACACAAA
ATTGTACCAT TCACATGACC TTTCACGTGA ACCTGCTTGG TCTCACATCG AATTGTGTTT

      1450        1460        1470        1480        1490        1500
GCACCCAACT TACACTTAGG AGATTTCAAC TTAACTTGAC CGCTCTGAGC TAAACCTAGC
CGTGGGTTGA ATGTGAATCC TCTAAAGTTG AATTGAACTG GCGAGACTCG ATTTGGATCG

      1510        1520        1530        1540        1550        1560
CCCAAACCCA CTCCACCTTA CTACCAGACA ACCTTAGCCA AACCATTTAC CCAAATAAAG
GGGTTTGGGT GAGGTGGAAT GATGGTCTGT TGGAATCGGT TTGGTAAATG GGTTTATTTC

      1570        1580        1590        1600        1610        1620
TATAGGCGAT AGAAATTGAA ACCTGGCGCA ATAGATATAG TACCGCAAGG GAAAGATGAA
ATATCCGCTA TCTTTAACTT TGGACCGCGT TATCTATATC ATGGCGTTCC CTTTCTACTT

      1630        1640        1650        1660        1670        1680
AAATTATAAC CAAGCATAAT ATAGCAAGGA CTAACCCCTA TACCTTCTGC ATAATGAATT
TTTAATATTG GTTCGTATTA TATCGTTCCT GATTGGGGAT ATGGAAGACG TATTACTTAA
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      1690        1700        1710        1720        1730        1740
AACTAGAAAT AACTTTGCAA GGAGAGCCAA AGCTAAGACC CCCGAAACCA GACGAGCTAC
TTGATCTTTA TTGAAACGTT CCTCTCGGTT TCGATTCTGG GGGCTTTGGT CTGCTCGATG

      1750        1760        1770        1780        1790        1800
CTAAGAACAG CTAAAAGAGC ACACCCGTCT ATGTAGCAAA ATAGTGGGAA GATTTATAGG
GATTCTTGTC GATTTTCTCG TGTGGGCAGA TACATCGTTT TATCACCCTT CTAAATATCC

      1810        1820        1830        1840        1850        1860
TAGAGGCGAC AAACCTACCG AGCCTGGTGA TAGCTGGTTG TCCAAGATAG AATCTTAGTT
ATCTCCGCTG TTTGGATGGC TCGGACCACT ATCGACCAAC AGGTTCTATC TTAGAATCAA

      1870        1880        1890        1900        1910        1920
CAACTTTAAA TTTGCCCACA GAACCCTCTA AATCCCCTTG TAAATTTAAC TGTTAGTCCA
GTTGAAATTT AAACGGGTGT CTTGGGAGAT TTAGGGGAAC ATTTAAATTG ACAATCAGGT

      1930        1940        1950        1960        1970        1980
AAGAGGAACA GCTCTTTGGA CACTAGGAAA AAACCTTGTA GAGAGAGTAA AAAATTTAAC
TTCTCCTTGT CGAGAAACCT GTGATCCTTT TTTGGAACAT CTCTCTCATT TTTTAAATTG

      1990        2000        2010        2020        2030        2040
ACCCATAGTA GGCCTAAAAG CAGCCACCAA TTAAGAAAGC GTTCAAGCTC AACACCCACT
TGGGTATCAT CCGGATTTTC GTCGGTGGTT AATTCTTTCG CAAGTTCGAG TTGTGGGTGA

      2050        2060        2070        2080        2090        2100
ACCTAAAAAA TCCCAAACAT ATAACTGAAC TCCTCACACC CAATTGGACC AATCTATCAC
TGGATTTTTT AGGGTTTGTA TATTGACTTG AGGAGTGTGG GTTAACCTGG TTAGATAGTG

      2110        2120        2130        2140        2150        2160
CCTATAGAAG AACTAATGTT AGTATAAGTA ACATGAAAAC ATTCTCCTCC GCATAAGCCT
GGATATCTTC TTGATTACAA TCATATTCAT TGTACTTTTG TAAGAGGAGG CGTATTCGGA

      2170        2180        2190        2200        2210        2220
GCGTCAGATT AAAACACTGA ACTGACAATT AACAGCCCAA TATCTACAAT CAACCAACAA
CGCAGTCTAA TTTTGTGACT TGACTGTTAA TTGTCGGGTT ATAGATGTTA GTTGGTTGTT

      2230        2240        2250        2260        2270        2280
GTCATTATTA CCCTCACTGT CAACCCAACA CAGGCATGCT CATAAGGAAA GGTTAAAAAA
CAGTAATAAT GGGAGTGACA GTTGGGTTGT GTCCGTACGA GTATTCCTTT CCAATTTTTT

      2290        2300        2310        2320        2330        2340
AGTAAAAGGA ACTCGGCAAA TCTTACCCCG CCTGTTTACC AAAAACATCA CCTCTAGCAT
TCATTTTCCT TGAGCCGTTT AGAATGGGGC GGACAAATGG TTTTTGTAGT GGAGATCGTA

      2350        2360        2370        2380        2390        2400
CACCAGTATT AGAGGCACCG CCTGCCCAGT GACACATGTT TAACGGCCGC GGTACCCTAA
GTGGTCATAA TCTCCGTGGC GGACGGGTCA CTGTGTACAA ATTGCCGGCG CCATGGGATT

      2410        2420        2430        2440        2450        2460
CCGTGCAAAG GTAGCATAAT CACTTGTTCC TTAAATAGGG ACCTGTATGA ATGGCTCCAC
GGCACGTTTC CATCGTATTA GTGAACAAGG AATTTATCCC TGGACATACT TACCGAGGTG

      2470        2480        2490        2500        2510        2520
GAGGGTTCAG CTGTCTCTTA CTTTTAACCA GTGAAATTGA CCTGCCCGTG AAGAGGCGGG
CTCCCAAGTC GACAGAGAAT GAAAATTGGT CACTTTAACT GGACGGGCAC TTCTCCGCCC
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      2530        2540        2550        2560        2570        2580
CATAACACAG CAAGACGAGA AGACCCTATG GAGCTTTAAT TTATTAATGC AAACAGTACC
GTATTGTGTC GTTCTGCTCT TCTGGGATAC CTCGAAATTA AATAATTACG TTTGTCATGG

      2590        2600        2610        2620        2630        2640
TAACAAACCC ACAGGTCCTA AACTACCAAA CCTGCATTAA AAATTTCGGT TGGGGCGACC
ATTGTTTGGG TGTCCAGGAT TTGATGGTTT GGACGTAATT TTTAAAGCCA ACCCCGCTGG

      2650        2660        2670        2680        2690        2700
TCGGAGCAGA ACCCAACCTC CGAGCAGTAC ATGCTAAGAC TTCACCAGTC AAAGCGAACT
AGCCTCGTCT TGGGTTGGAG GCTCGTCATG TACGATTCTG AAGTGGTCAG TTTCGCTTGA

      2710        2720        2730        2740        2750        2760
ACTATACTCA ATTGATCCAA TAACTTGACC AACGGAACAA GTTACCCTAG GGATAACAGC
TGATATGAGT TAACTAGGTT ATTGAACTGG TTGCCTTGTT CAATGGGATC CCTATTGTCG

      2770        2780        2790        2800        2810        2820
GCAATCCTAT TCTAGAGTCC ATATCAACAA TAGGGTTTAC GACCTCGATG TTGGATCAGG
CGTTAGGATA AGATCTCAGG TATAGTTGTT ATCCCAAATG CTGGAGCTAC AACCTAGTCC

      2830        2840        2850        2860        2870        2880
ACATCCCGAT GGTGCAGCCG CTATTAAAGG TTCGTTTGTT CAACGATTAA AGTCCTACGT
TGTAGGGCTA CCACGTCGGC GATAATTTCC AAGCAAACAA GTTGCTAATT TCAGGATGCA

      2890        2900        2910        2920        2930        2940
GATCTGAGTT CAGACCGGAG TAATCCAGGT CGGTTTCTAT CTACCTTCAA ATTCCTCCCT
CTAGACTCAA GTCTGGCCTC ATTAGGTCCA GCCAAAGATA GATGGAAGTT TAAGGAGGGA

      2950        2960        2970        2980        2990        3000
GTACGAAAGG ACAAGAGAAA TAAGGCCTAC TTCACAAAGC GCCTTCCCCC GTAAATGATA
CATGCTTTCC TGTTCTCTTT ATTCCGGATG AAGTGTTTCG CGGAAGGGGG CATTTACTAT

      3010        3020        3030        3040        3050        3060
TCATCTCAAC TTAGTATTAT ACCCACACCC ACCCAAGAAC AGGGTTTGTT AAGATGGCAG
AGTAGAGTTG AATCATAATA TGGGTGTGGG TGGGTTCTTG TCCCAAACAA TTCTACCGTC

      3070        3080        3090        3100        3110        3120
AGCCCGGTAA TCGCATAAAA CTTAAAACTT TACAGTCAGA GGTTCAATTC CTCTTCTTAA
TCGGGCCATT AGCGTATTTT GAATTTTGAA ATGTCAGTCT CCAAGTTAAG GAGAAGAATT

      3130        3140        3150        3160        3170        3180
CAACATACCC ATGGCCAACC TCCTACTCCT CATTGTACCC ATTCTAATCG CAATGGCTGA
GTTGTATGGG TACCGGTTGG AGGATGAGGA GTAACATGGG TAAGATTAGC GTTACCGACT

      3190        3200        3210        3220        3230
TCATTTGTTA AGATGGCAGA GCCCGGTAAT CGCATAAAAT GAGACCGGAT CC
AGTAAACAAT TCTACCGTCT CGGGCCATTA GCGTATTTTA CTCTGGCCTA GG
```
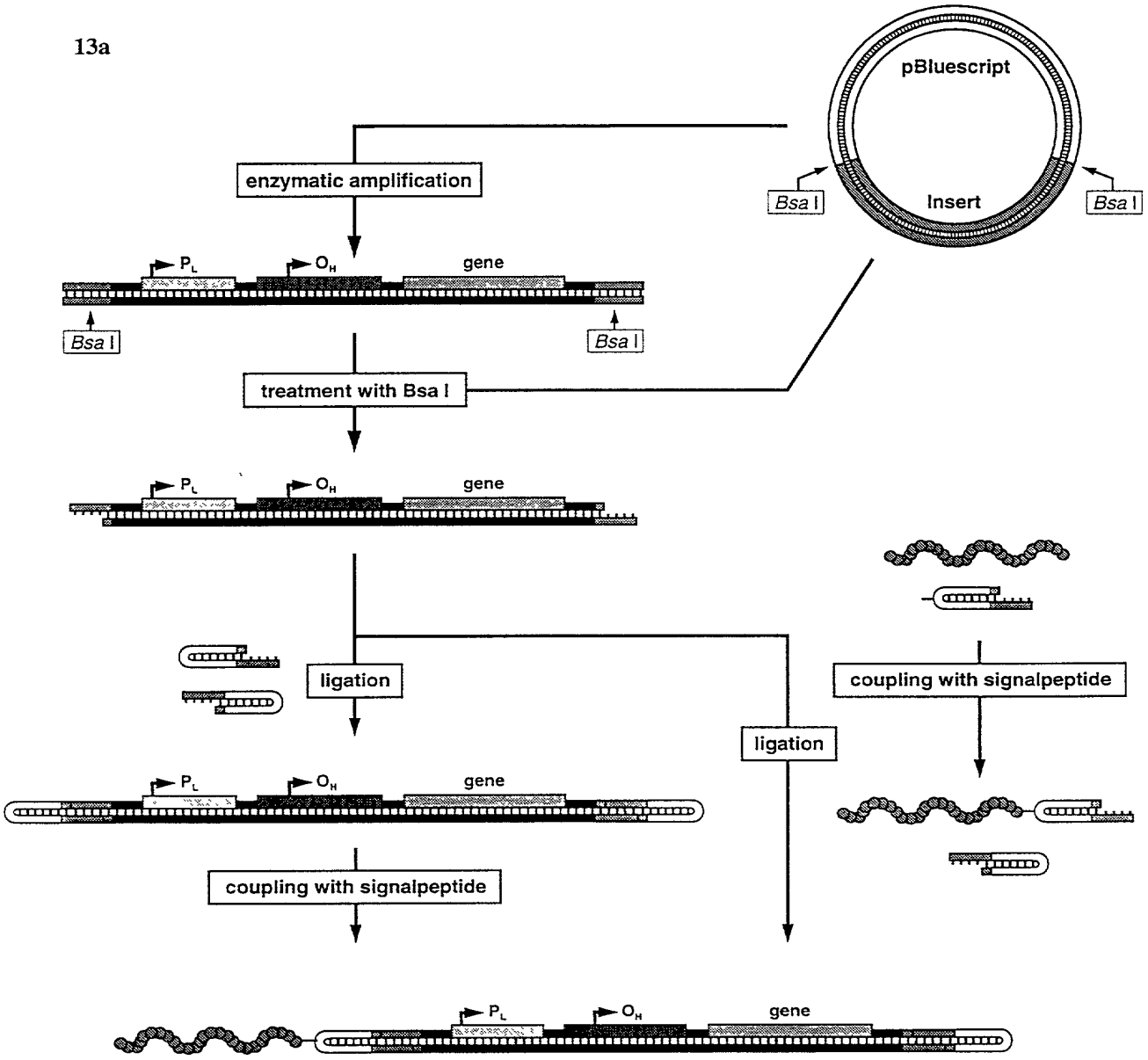

Figure 13

13a



13b

CCCCGGGTACCTTGCGAGCCC$_X$
CCCATGGAACGCTCGGG

HP 1 (X=modified dT)

TTTTGCAGCTGGATCCCGGGC$_A$
CGTCGACCTAGGGCCCG

HP 2

Figure 14

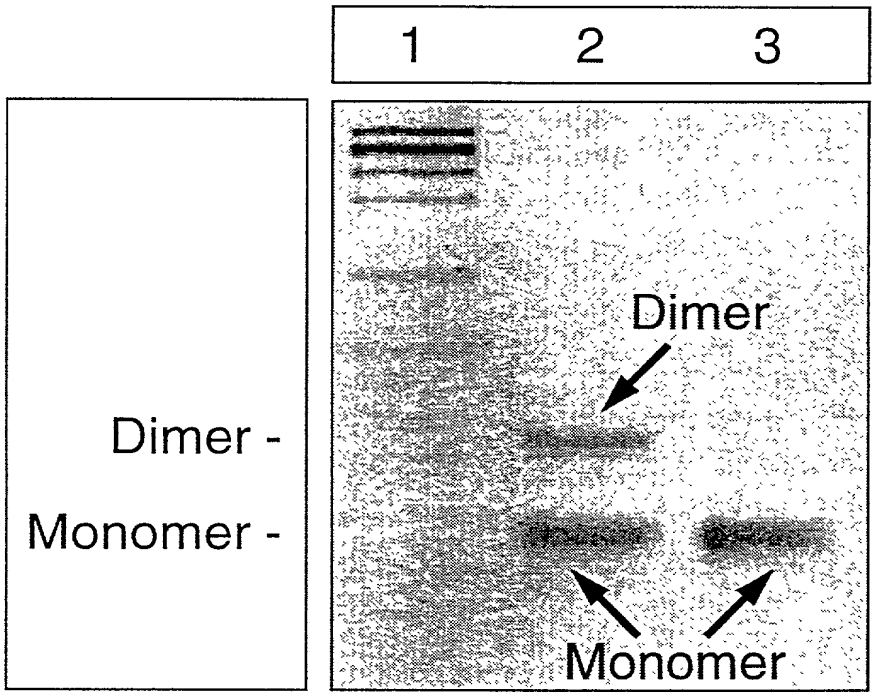
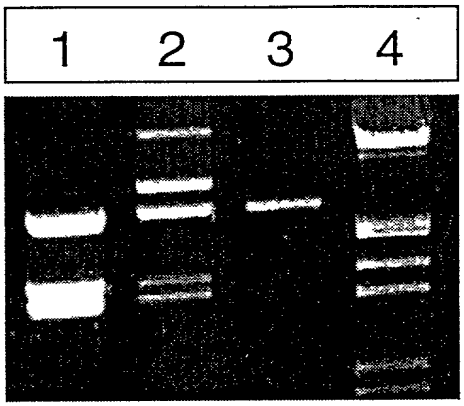


Figure 15

15a



15b

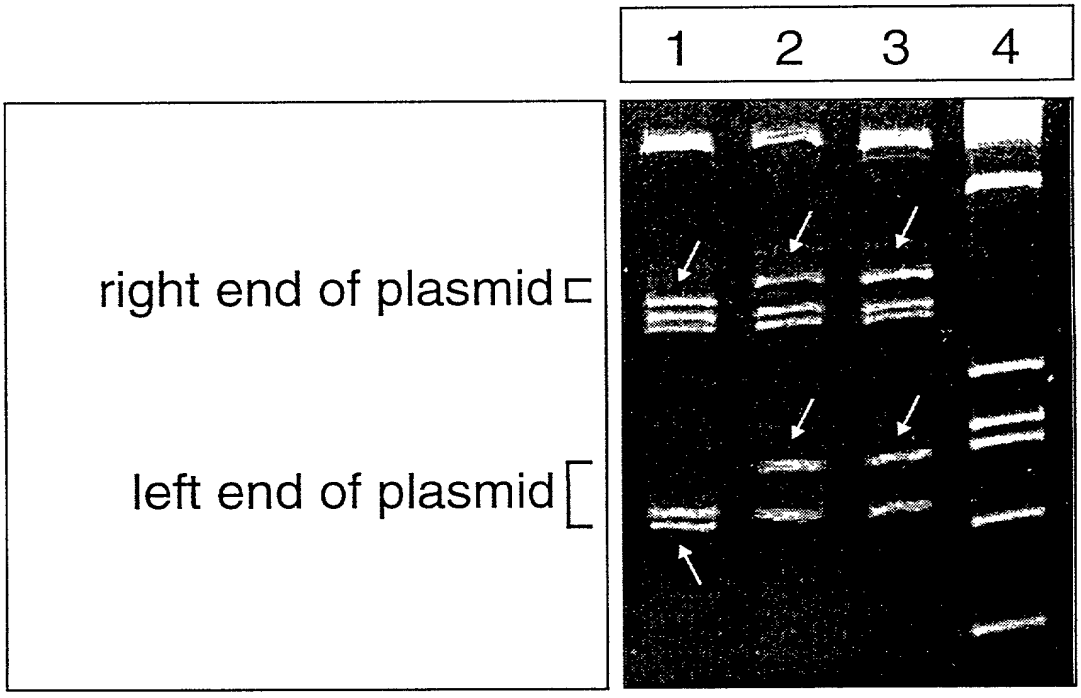
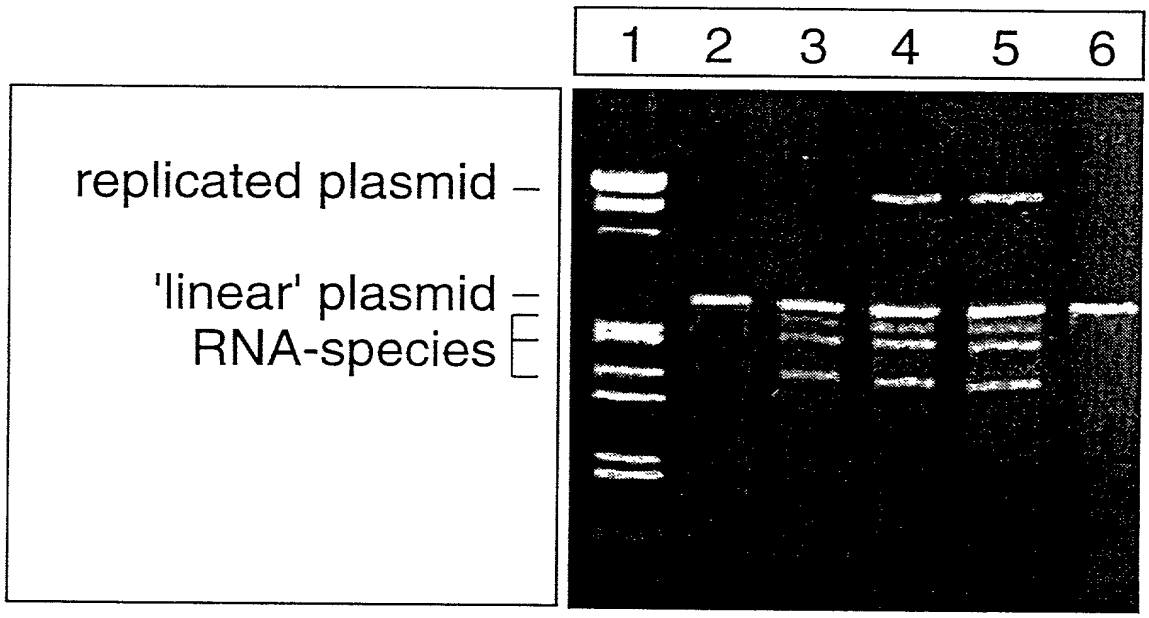


Figure 16



PENNIE & EDMONDS DOCKET NO. 8484-018-999

DECLARATION
AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. underneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

Chimerical Peptide-Nucleic Acid Fragment, Process For Producing The Same And Its Uses For Appropriately Introducing Nucleic Acids Into Cell Organelles and Cells

and for which a patent application:
☐ is attached hereto and includes amendment(s) filed on _____ *(if applicable)*
☒ was filed in the United States on December 16, 1996 as Application Serial No. _____ *(for declaration not accompanying application)*
with amendment(s) filed on December 16, 1996 *(if applicable)*
☒ was filed as PCT international application Serial No. PCT/DE95/00775 on June 11, 1995 and was amended under PCT Article 19 on _____ *(if applicable)*

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
P 44 21 079.5	GERMANY	16/06/94	YES ☒ NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint S. Leslie Misrock (Reg. No. 18872), Harry C. Jones, III (Reg. No. 20280), Berj A. Terzian (Reg. No. 20060), Gerald J. Flintoft (Reg. No. 20823), David Weild, III (Reg. No. 21094), Jonathan A. Marshall (Reg. No. 24614), Barry D. Rein (Reg. No. 22411), Stanton T. Lawrence, III (Reg. No. 25736), Isaac Jarkovsky (Reg. No. 22713), Joseph V. Colaianni (Reg. No. 20019), Charles E. McKenney (Reg. No. 22795), Philip T. Shannon (Reg. No. 24278), Francis E. Morris (Reg. No. 24615), Charles E. Miller (Reg. No. 24576), Gidon D. Stern (Reg. No. 27469), John J. Lauter, Jr. (Reg. No. 27814), Brian M. Poissant (Reg. No. 28462), Brian D. Coggio (Reg. No. 27624), Rory J. Radding (Reg. No. 28749), Stephen J. Harbulak (Reg. No. 29166), Donald J. Goodell (Reg. No. 19766), James N. Palik (Reg. No. 25510), Thomas E. Friebel (Reg. No. 29258), Laura A. Coruzzi (Reg. No. 30742), Jennifer Gordon (Reg. No. 30753), Jon R. Stark (Reg. No. 30111), Allan A. Fanucci (Reg. No. 30256), Geraldine F. Baldwin (Reg. No. 31232), Victor N. Balancia (Reg. No. 31231), Albert P. Halluin (Reg. No. 25227), Samuel B. Abrams (Reg. No. 30605), Steven I. Wallach (Reg. No. 35402), Marcia H. Sundeen (Reg. No. 30893), Paul J. Zegger (Reg. No. 33821), Edmond R. Bannon (Reg. No. 32110), Bruce J. Barker (Reg. No. 33291), Adriane M. Antler (Reg. No. 32605), Ann L. Gisolfi (Reg. No. 31956), SaraLynn Mandel (Reg. No. 31853), Mark A. Farley (Reg. No. 33170), James G. Markey (Reg. No. 31636), and Charles F. Hoyng (Reg. No. 35548), all of Pennie & Edmonds, whose addresses are 1155 Avenue of the Americas, New York, New York 10036, 1667 K Street N.W., Washington, DC 20006 and 2730 Sand Hill Road, Menlo Park, CA 94025, and each of them, my attorneys, to prosecute this application, and to transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201 - Peter Seibel	SIGNATURE OF INVENTOR 202 - Andrea Seibel
DATE 21.3.97	DATE 21.3.97